REMARKS

Claims 9-13 are pending in this application. Claims 1-8 have been canceled, without prejudice or disclaimer. As to new claims 12-13, see original claim 9.

In the office action, Claims 1-11 have been rejected for nonstatutory obviousness type double patenting based on claims 3-10 of U.S. Patent No. 5,293,772 in view of Ramsis et al. (Abstract). Claims 1-11 also have been rejected for nonstatutory obviousness-type double patenting as being unpatentable over claims 3-10 of U.S. Patent No. 5,205,159 in view of Ramsis et al. Claims 1-11 also have been rejected for nonstatutory obviousness-type double patenting based on claims 7-15 of U.S. Patent No. 4,986,964 in view of Ramsis et al.

At page 7 of the office action, Claims 1-11 have been rejected under 35 U.S.C. 103(a) as being unpatentable over Carr (US 5,293,772) in view of Ramsis et al. At page 9 of the office action, Claims 1-11 have been rejected under 35 U.S.C. 103(a) as being unpatentable over Carr (US 5,205,159) in view of Ramsis et al. At page 10 of the office action, Claims 1-11 have been rejected under 35 U.S.C. 103(a) as being unpatentable over Carr et al. (US 4,986,964) in view of Ramsis et al.

Applicants respectfully traverse these rejections.

Applicants' presently claimed invention is a method of monitoring treatment or therapy of a patient suffering from unstable angina or myocardial infarction. (Claim 9.) Applicants' inventive method comprises steps of obtaining a baseline measurement (platelet contractile force or clot elastic modulus) on a blood sample taken from the patient; providing the patient with treatment or therapy; obtaining a platelet contractile force or clot elastic modulus measurement on the blood sample after the providing step; and comparing that measurement and the baseline measurement, wherein progress of the treatment or therapy is indicated by a decline in the subsequent measurement relative to the baseline measurement. (Applicants' claim 9.)

Applicants' presently claimed invention is substantially removed from the three Carr patents cited by the Examiner. The earliest of the cited patents, Carr '964, does not disclose angina and only mentions myocardial infarctions in the Background of the invention (col. 2, line 42) along with cerebral vascular accidents as direct results of inappropriate clot deposition. The instrumentation disclosed in Carr '964 was designed to address a need "for a simple, low cost

instrument which can help define the interactions between platelets and fibrin and to assess how these interactions impact on the eventual clot removal." (Col. 2, lines 49-53.) Neither of the two subsequent Carr patents '772, '159 discloses monitoring treatment or therapy of a patient suffering from angina or myocardial infarction or even mentions angina or myocardial infarction. To arrive at Applicants' presently claimed inventive methods of monitoring treatment or therapy of a patient suffering from unstable angina or myocardial infarction was beyond what a person of ordinary skill in Applicants' art could have accomplished, because even with the three Carr patents and Ramsis, that person of ordinary skill in the art would not have arrived at Applicants' presently claimed invention.

Ramsis would not have been used by a person of ordinary skill in the art to modify the Carr patents as the Examiner hypothsizes. Ramsis (1998) only discloses a one time measurement using flow cytometry. Ramsis is measuring baseline activation not total activation as in Applicants' claimed invention. Additionally, flow cytometry (used by Ramsis) must be performed in a system without thrombin. Measuring platelet contractile force (as recited in Applicants' claims 9, 10) necessarily requires presence of thrombin. Ramsis and the Carr patents are not combinable as the Examiner proposes.

The person of ordinary skill in the art would have needed more than the three Carr patents plus Ramsis. Without Applicants' present specification filed April 17, 2002 or the later articles (such as Carr, "Development of Platelet Contractile Force as a Research and Clinical Measure of Platelet Function," *Cell Biochemistry and Biophysics,* 38:55-78 (2003); Carr, et al., "Effect of Non-Heparin Thrombin Antagonists on Thrombin Generation, Platelet Function, and Clot Structure in Whole Blood," *Cell Biochemistry and Biophysics,* 39:89-99 (2003) submitted herewith as attachments), a person of ordinary skill in the art would have had too little to arrive at Applicants' presently claimed invention.

Wherefore, reconsideration and withdrawal of the obviousness-type double patenting rejections and the obviousness rejections are respectfully sought.

In view of the foregoing, it is respectfully requested that the application be reconsidered, that claims 9-13 be allowed, and that the application be passed to issue.

Should the Examiner find the application to be other than in condition for allowance, the Examiner is requested to contact the undersigned at the local telephone number listed below to

discuss any other changes deemed necessary in a telephonic or personal interview.

A provisional petition is hereby made for any extension of time necessary for the continued pendency during the life of this application. Please charge any fees for such provisional petition and any deficiencies in fees and credit any overpayment of fees to Attorney's Deposit Account No. 50-2041 (Whitham, Curtis & Christofferson, P.C.).

Respectfully submitted,

Mary E. Goulet
Reg. No. 35,884

Whitham, Curtis
Christofferson & Cook, P.C.
11491 Sunset Hills Road, Suite 340
Reston, VA 20190

Tel. (703) 787-9400
Fax. (703) 787-7557

Customer No.: 30743

Attachment 1
(10/049,374)

1085-9195/03/39/89–98/$25.00

ORIGINAL ARTICLE

Effect of Non-Heparin Thrombin Antagonists on Thrombin Generation, Platelet Function, and Clot Structure in Whole Blood

Marcus E. Carr, Jr.,[1–5] *Pantep Angchaisuksiri,*[3,4] *Sheryl L. Carr,*[1,4] *and Erika J. Martin*[1,4]

[1]*Coagulation Special Studies Laboratory,* [2]*Division of Hematology/Oncology,* [3]*Departments of Medicine and Pathology, Medical College of Virginia;* [4]*Virginia Commonwealth University, and* [5]*Richmond Veterans Administration Medical Center, Richmond, VA 23298-0230*

Abstract

Platelet contractile force (PCF), which is absent in blood obtained during cardiopulmonary bypass, significantly recovers after protamine sulfate administration. In vitro studies reveal this effect to be primarily caused by heparin. Because many of heparin's effects are mediated by suppression of thrombin generation and activity, this study assessed the influence of thrombin inhibition on PCF. The effects of natural and synthetic antithrombins were measured. Clots were formed by the addition of batroxobin (0.21 µg/mL) to whole blood (platelet count 200,000/µL). Force development was measured from the moment of batroxobin addition. After 1200 s of clotting, purified antithrombin III (22 µ*M*) reduced PCF by 74%. Thrombomodulin (0.014 µ*M*) reduced PCF by 60%. At 0.040 µ*M*, PCF was reduced by 82% (6.5–1.2 Kdynes). Hirudin decreased PCF in a dose-dependent fashion, with complete suppression at concentrations ≥ 0.30 µ*M*. At concentrations between 0.04 and 0.29 µ*M*, Lepirudin (Refludan, a recombinant therapeutic hirudin) produced dose-dependent delay and suppression of PCF. Above 0.29 µ*M* Lepirudin, PCF was totally suppressed. At 1.60 µ*M*, bivalirudin (a synthetic, 20 amino acid peptide) delayed and reduced PCF by 50%. At 6.40 µ*M*, PCF was completely suppressed. Although 20 µ*M* of P-PACK II (d-Phenylalanyl-L-Phenylalanyl-arginine-chloro-methyl ketone 2 HCl) had little effect, 40 µ*M* delayed onset of force development from 300 to 600 s and reduced PCF at 1200 s from 5.2 to 3.3 Kdynes. At 120 µ*M*, force development was totally suppressed. Four micromol Thromstop® (BNas-Gly-(pAM)Phe-Pip) delayed force development by greater than 800 s and PCF at 1200 s was reduced by 70%. At 0.20 µ*M*, Argatroban (a synthetic polypeptide direct thrombin antagonist) delayed onset of PCF from 300 to 540 s and decreased PCF by 40%. At a concentration of 0.40 µ*M* and above, Argatroban totally suppressed PCF. These results indicate that some of the antiplatelet effects of heparin are the result of thrombin inhibition and that low-level thrombin generation is essential for clot retraction. The sensitivity of PCF to the presence of thrombin may permit monitoring of antithrombin agents via this assay.

Index Entries: Antithrombin III; thrombomodulin; hirudin; lepirudin; bivalirudin; thromstop; P-PACK II; argatroban.

*Author to whom all correspondence and reprint requests should be addressed: Department of Medicine, Box 980230, Virginia Commonwealth University, Richmond, VA 23298-0230. E-mail: mcarr@hsc.vcu.edu.

INTRODUCTION

When vascular injury occurs, a complex sequence of cellular and extracellular events are initiated that result in rapid plugging of the vascular leak via the production of a clot. Cellular processes include adhesion of platelets to the site of injury, recruitment of additional platelets to the platelet plug, and provision of an appropriate membrane surface for activation of plasma clotting factors *(1)*. Extracellular protein events include adhesion and activation of contact factors *(2)*, activation of clotting factors via exposure of tissue factor *(3)*, and the subsequent activation of the potent serine protease thrombin *(4,5)*. The production of thrombin is a critical event. Once activated, thrombin is a profound activator of platelets *(6)*, is the sole enzyme responsible for fibrin production *(7)*, and is a potent positive feedback signal for additional thrombin production *(8)*.

To prevent dissemination of the clotting event, thrombin that escapes the clot is rapidly neutralized either by binding to antithrombin III *(9)* or by binding to thrombomodulin on endothelial cell surfaces *(10)*. Although development of plasma thrombin coagulant activity is easily assayed by clotting tests such as the prothrombin time *(11)* and the activated partial thromboplastin time *(12)*, the effects of thrombin on platelet function are not routinely measured or considered.

A new instrument is now available that allows simultaneous measurement of platelet function and clot structure during clotting of whole blood or platelet-rich plasma *(13)*. Platelet function is monitored as the time dependent generation of platelet contractile force (PCF measured in Kdynes) during clot retraction *(14,15)*. Clot formation and structure are measured as time-dependent increases in clot elastic modulus (CEM expressed in Kdynes/cm^2) *(16,17)*. PCF is principally a measure of platelet function *(18)*. Blockade of the platelet fibrinogen receptor GP IIb/IIIa *(19)*, increased intracellular cyclic adenosine monophosphate (cAMP) *(20,21)*, or decreased platelet concentration all reduce force generation *(14)*. CEM depends on both platelet function and fibrin structure *(16,22)*. The combination of PCF and CEM measurements aids in the differentiation of abnormal platelet function from abnormal clot formation *(18)*.

PCF is thrombin-dependent. If blood is clotted by the addition of snake venom enzymes such as reptilase or batroxobin, clot formation occurs, but force is not generated *(23,24)*. If a sample of citrated whole blood is clotted by the addition of batroxobin and calcium, PCF begins to develop after a lag phase during which thrombin is generated by the recalcification of the system. Thus, the onset of PCF development can serve as a surrogate marker of thrombin generation in whole blood *(25)*. This assay is sensitive to deficient thrombin generation due to a variety of clotting factor deficiencies *(25)* and to inhibition of coagulation by heparin *(26)*. In this study, we document the effects of a variety of non-heparin antithrombins on PCF and CEM.

MATERIALS AND METHODS

Sample Preparation

Blood was obtained from a healthy volunteer via aseptic venipuncture into evacuated tubes containing 3.2% sodium citrate. The studies of the effects of non-heparin thrombin antagonists on PCF and CEM were performed on whole blood (platelet count, 200,000/μL).

Materials

Batroxobin, purchased as Atroxin®, *Bothrops atrox* venom protein (Sigma Diagnostics, St. Louis, MO), was dissolved in deionized water, and used without further purification. Atroxin was maintained on ice and used within 3 h of being dissolved. Refludan® (lepirudin [recombinant deoxyribonucleic acid; rDNA]) was obtained from Hoechst Marion Roussel, Inc. (Kansas City, MO). Argatroban was obtained from Smith Kline Beecham Pharmaceuticals (Philadelphia, PA). Purified human antithrombin III (Thrombate III™) was obtained from Bayer Pharmaceuticals (Westhaven, CT). Thrombomodulin was purchased as a purified

material from American Diagnostica Inc. (Greenwich, CT). P-PACK II(r) (d-Phenylalanyl-L-Phenylalanyl-arginine-chloro-methyl ketone 2 HCl), a synthetic serine protease inhibitor that inhibits contact pathway activation at low concentrations (50 nm) (*27*) and directly inhibits thrombin at high concentrations such as those used in the present study (*28*), was purchased as a purified powder from Calbiochem (La Jolla, CA). Thromstop® (BNas-Gly-(pAM)Phe-Pip), another synthetic antithrombin, was purchased from American Diagnostica, Inc. (Greenwich, CT). Hirudin from leeches was purchased from Sigma Diagnostics (St. Louis, MO). Bivalirudin was obtained from The Medicines Company (Cambridge, MA). Calcium chloride was purchased as analytical grade purified salts. Stock solutions were prepared using deionized, distilled water.

Measurement of Force Development and Clot Elastic Modulus

Forces generated by platelets were measured with the Hemodyne Hemostasis Analyzer (Hemodyne, Inc., Richmond, VA). Clots are formed between a temperature-controlled cup and a parallel upper surface (*14,15*). Before clotting, the upper surface is centered above the cup and lowered into the clotting solution. As the clot forms, it attaches to the inner walls of the cup and upper surface. Once clotting is complete, platelets within the network pull fibrin strands inward transmitting force through the network to the surfaces to which the clot is adherent. Force measurement is accomplished using a displacement transducer coupled to the upper surface. As platelets contract, the transducer produces an electrical output proportional to the amount of force generated.

Clot elastic modulus was measured simultaneously with PCF. A downward force of known magnitude is applied to the upper surface and the amount of downward displacement is measured (*29*). The ratio of downward force per unit area of application (stress) to downward displacement (strain) is used to calculate the elastic modulus. Kinetics of modulus development during clotting were monitored by repeatedly stressing the network with a standard force.

All measurements were done in duplicate and are presented as the mean ± the standard error of the mean.

Thrombin Generation Time Determination

Thrombin generation time (TGT) was determined from the lag phase before PCF development. Citrated blood clotted with batroxobin and calcium develops PCF. Because batroxobin does not activate platelets, there is a lag phase before the onset of PCF. During this time, thrombin is generated by recalcification. Because the fibrin network is in place prior to the generation of thrombin, PCF becomes apparent as soon as a small amount of thrombin is generated. Thus, the inflection or take off point in the PCF curve serves as a marker of thrombin generation in the batroxobin mediated assay (*30,31*).

Fibrin Mass/Length Ratio Determination

Platelet-poor plasma was prepared by centrifugation at 1500*g* for 15 min. The fibrinogen concentration of plasma samples was determined by the modified method of Clauss (*32*). Plasma gels were formed directly in 10-mm polystyrene cuvettes (Fisher Scientific Co., Pittsburgh, PA) by adding 0.8 mL of citrated plasma to 1.2 mL of buffered batroxobin (0.21 μg/mL). Turbidity measurements were made utilizing a thermostated Schimadzu spectrophotometer. Turbidity was monitored for 30 min, after which gelation was allowed to go to completion unobserved. After 24 h, the gels were scanned from 400 to 800 nm and the mass/length ratios of the fibrin fibers were determined according to the following equation (*33,34*):

$$\tau = ((88/15)\pi^3 n(dn/dC)^2 C\mu)/N\lambda^3$$

where τ is the turbidity, n is the solution refractive index, dn/dC the refractive index increment, λ the wavelength, C the concentration of fibrinogen in g/mL, N Avogadro's number, and μ the mass/length ratio. For clear gels, μ was

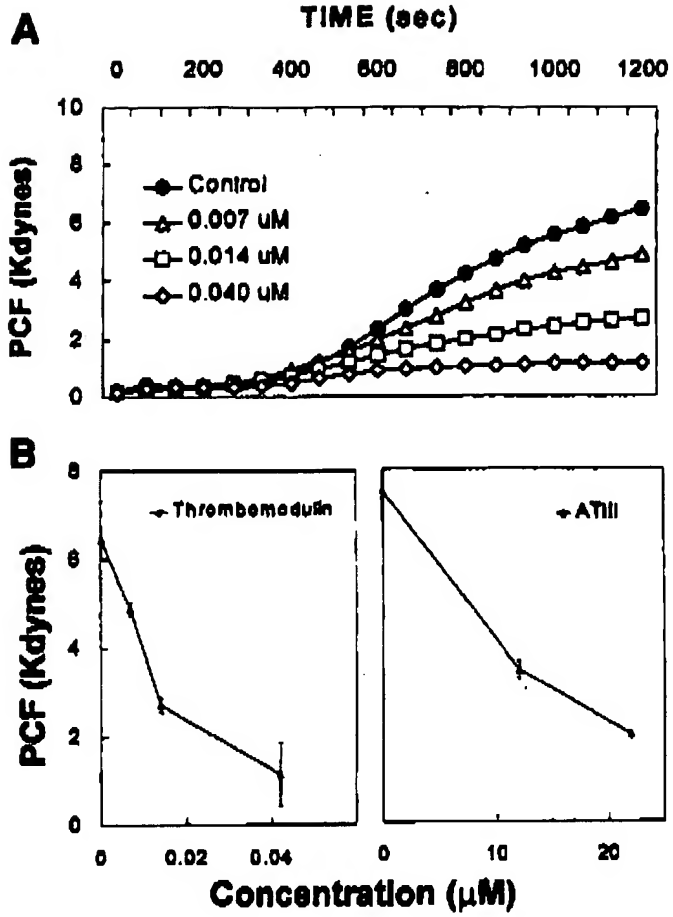

Fig. 1. **A**: Effect of increasing concentrations of purified thrombomodulin on the kinetics of platelet contractile force (PCF) development in whole blood clotted by the addition of batroxobin (0.21 μg/mL) and calcium (10 mmol/L final concentration) at time zero. **B**: Effect of increasing thrombomodulin concentration (closed triangles) and increasing antithrombin III concentration (open triangles) on platelet contractile force (PCF). Results are plotted as the mean ± SEM of duplicate measurements.

determined from the slope of a plot of τ vs $1/\lambda^3$. For more turbid gels, where the radius of the fibers is no longer small relative to the incident wavelength, μwas obtained from the inverse of the intercept of a plot of $C/\tau\, z\lambda^3$ vs $1/\lambda^2$ (*35*).

RESULTS

One minute before the addition of calcium (10 m*M*, final) and batroxobin (0.21 μ/mL), increasing concentrations of antithrombins were added to 700 μL of citrated (3.2%) whole blood. The kinetics of PCF and CEM development were observed for 1200 s.

Physiologic Antithrombins

The effect of purified human antithrombin III on platelet mediated force development is illustrated in Fig. 1B. The addition of 12 μ*M* of antithrombin III decreased PCF by 55% and 22

μ*M* decreased PCF by 74%. The decreases in PCF were accompanied by a remarkable lengthening of the lag phase.

Thrombomodulin, another specific protein binder of thrombin, proved to be an effective inhibitor of force development (Fig. 1A, B). The control clot (upper curve, Fig. 1A) was formed by the addition of batroxobin at time equal zero. After 300 s, clotting had proceeded to a point that allowed transmission of force through the fibrin network. Over the next 900 s, force development continued, reaching 6.5 Kdynes by 1200 s. Addition of 0.014 μ*M* of thrombomodulin per milliliter of clotting mixture decreased PCF by 60%. Tripling the thrombomodulin concentration delayed the onset of force development by 100 s and resulted in a 82% reduction of PCF at 1200 s. The effects of these physiologic antithrombins were also reflected in changes in CEM (Table 1).

Hirudin-Like Antithrombins

The effects of hirudin on platelet force development are depicted in Fig. 2B. Hirudin concentrations between 0 and 0.30 μ*M* lead to dose dependent suppression of force development. Force at 1200 s dropped from 6.5 to 4.0 to 3.6 to 1.8 Kdynes as the hirudin concentration increased from 0–0.04 to 0.08–0.015 μ*M*. At 0.30 μ*M*, force development was absent. Lepirudin, a highly specific thrombin inhibitor, proved to be an effective inhibitor of PCF (Fig. 2A, B). After 300 s, clot structure of the control clot (closed circles) was sufficient for force transmission. At lepirudin concentrations between 0.04 and 0.29 μ*M* dose-dependent suppression of PCF occurred. At 0.29 μ*M*, force development was absent. At 1.60 μ*M*, bivalirudin (a synthetic, 20 amino acid peptide) delayed and reduced PCF by 50%. At 6.40 μ*M*, PCF was completely suppressed. The effects of these hirudin-like antithrombins were also reflected in changes in CEM (*see* Table 1).

Synthetic Antithrombins

The effect of P-PACK-II, on PCF is demonstrated in Fig. 3B. At P-Pack II concentrations above 20 μ*M*, a dose-dependent suppression of force development occurred. At 20 μ*M*, P-PACK-II should significantly inhibit the serine proteases of the contact/intrinsic pathway. The limited impact at this concentration, would indicate that much of the thrombin being generated in the system is via VIIa-platelet interactions. Above 20 μ*M*, P-PACK-II prolonged the lag-phase before initial force development and decreased the total force developed at 1200 s. Because P-PACK II also inhibits VIIa, the relative contributions of VIIa and thrombin inhibition cannot be separated. When compared to Thromstop® and Argatroban® (lower panel of Fig. 3), P-PACK II is seen to be a significantly weaker inhibitor of thrombin generation. The effect of Thromstop is depicted in Fig. 3B. At 4 μ*M*, Thromstop delayed initial force development by greater than 800 s. The force at 1200 s was reduced by 70%. The effect Argatroban is depicted in Fig. 3A, B. Argatroban concentrations between 0 and 0.2 μ*M* lead to dose dependent suppression of force development. PCF at 1200 seconds dropped from 6.1 to 5.0 to 3.8 Kdynes. At 0.2 μ*M* force development was absent. The effects of these synthetic antithrombins were also reflected in changes in CEM (*see* Table 1).

The effects of all studied antithrombins on the rate of thrombin generation (TGT) in whole blood are summarized in Table 1. Physiologic antithrombins (antithrombin III and thrombomodulin) prolonged the TGT in a dose-dependent fashion, but did not totally suppress thrombin generation even at significantly elevated concentrations. In contrast, hirudin-like and synthetic antithrombins (with the exception of Thromstop) were able to completely suppress thrombin generation.

The antithrombins used in this study had virtually no effect on early fibrin assembly in plasma clots as demonstrated by absorbance at 1200 s after the addition of batroxobin and calcium chloride (Table 2). As a result of the possibility of delayed additional network alteration by thrombin formed subsequent to 1200 s, clots were allowed to form for 24 h before structural analysis. Consistent with the analysis at 1200 s,

Table 1
Effect of Antithrombins on Clot Elastic Modulus and Thrombin Generation Time

	Added inhibitor (μM)	CEM at 1200 s (Kdynes/cm^2)	TGT (min)
Baseline	0	17.17 ± 0.55	5 to 6
Physiologic antithrombins			
AT III	12	13.5 ± 1.81	8
(MW = 58,000)	22	3.2 ± 0.01	12
Thrombomodulin	0.007	12.8 ± 0.92	7
(MW = 74,000)	0.014	6.8 ± 0.30	8
	0.042	1.98 ± 1.98	8.5
Hirudin-like antithrombins			
Hirudin	0.04	12.3 ± 1.76	7
(MW = 10,000)	0.08	11.2 ± 2.11	8
	0.15	6.5 ± 2.07	9
	0.30	0.7 ± 0.73	>20
Lepirudin	0.04	14.7 ± 1.42	8
(MW = 6,979.5)	0.07	9.0 ± 0.85	9
	0.14	7.2 ± 0.91	12
	0.29	3.6 ± 1.53	14
	0.57	0.03 ± 0.03	>20
Bivalirudin	0.8	10.93 ± 0.34	7
(MW = 2,180)	1.6	8.68 ± 0.98	9
	3.2	2.58 ± 0.82	14
	6.4	0	>20
Synthetic antithrombins			
P-Pack	20	13.21 ± 0.14	8
(MW = 573.95)	40	8.31 ± 0.23	11
	80	2.8 ± 2.01	11
	120	0	>20
Thromstop	1.0	14.3 ± 0.94	7
(MW = 581.7)	2.0	10.4 ± 0.22	10
	3.0	6.7 ± 0.72	10
	4.0	4.61 ± 0.5	13
Argatroban	0.1	14.8 ± 2.01	8
(MW = 526.66)	0.2	10.9 ± 1.91	11
	0.4	1.2 ± 0.05	>20
	0.8	0.5 ± 0.25	>20

Results are mean clot elastic modulus (CEM) and ± standard error of the mean (SEM). MW, molecular weight.

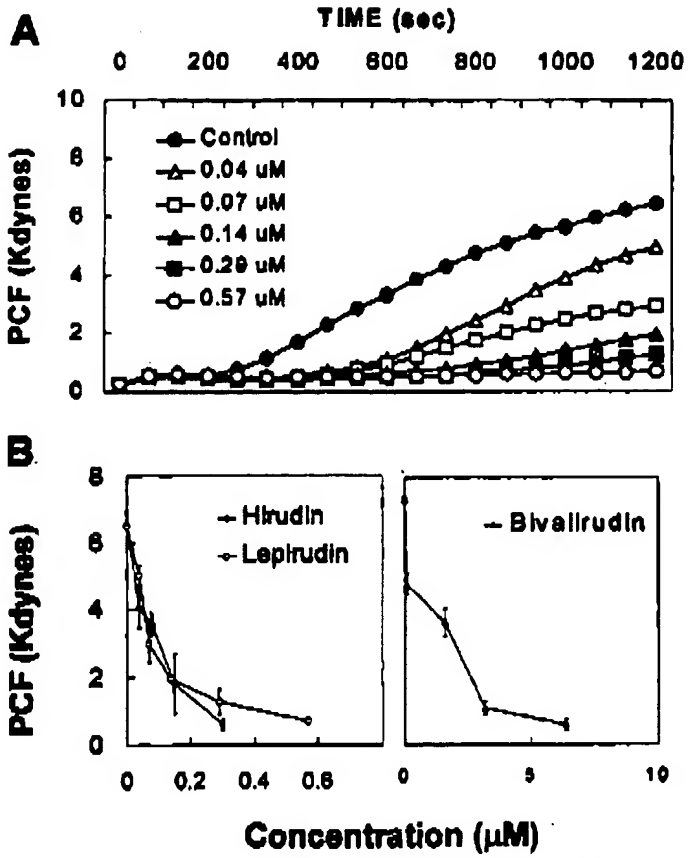

Fig. 2. **A**: Effect of increasing concentrations of lepirudin on the kinetics of platelet contractile force (PCF) development in whole blood clotted by the addition of batroxobin (0.21 μg/mL) and calcium (10 mmol/L final concentration) at time zero. **B**: Effect of increasing hirudin concentration (closed circles), increasing lepirudin concentration (open circles), and increasing bivalirudin concentration (closed triangles) on platelet contractile force (PCF). Results are plotted as the mean ± SEM of duplicate measurements.

fiber mass/length ratios for clots containing antithrombin III (12 μ*M*), thrombomodulin (0.04 μ*M*), hirudin (0.3 μ*M*), lepirudin (0.57 μ*M*), bivalirudin (6.40 μ*M*), thromstop (4 μ*M*), argatroban (0.2 μ*M*), and P-Pack II (120 μ*M*) did not differ from those of the control (*see* Table 2).

DISCUSSION

Low-level thrombin activity is essential for platelet-mediated force development (*23,24*). Suppression of thrombin activity leads to decreased PCF (*26*). Investigations in artificial plasma model systems have demonstrated the effects of various factor deficiencies on thrombin formation (*36,37*). We confirmed these findings in whole blood from patients with inherited clotting factor deficiencies (*25*). We have also noted delayed and decreased PCF in patients with warfarin induced factor deficiencies (*30*). Heparin reduces PCF in a dose-dependent fashion (*26*), and adequate reversal of heparin with protamine sulfate leads to

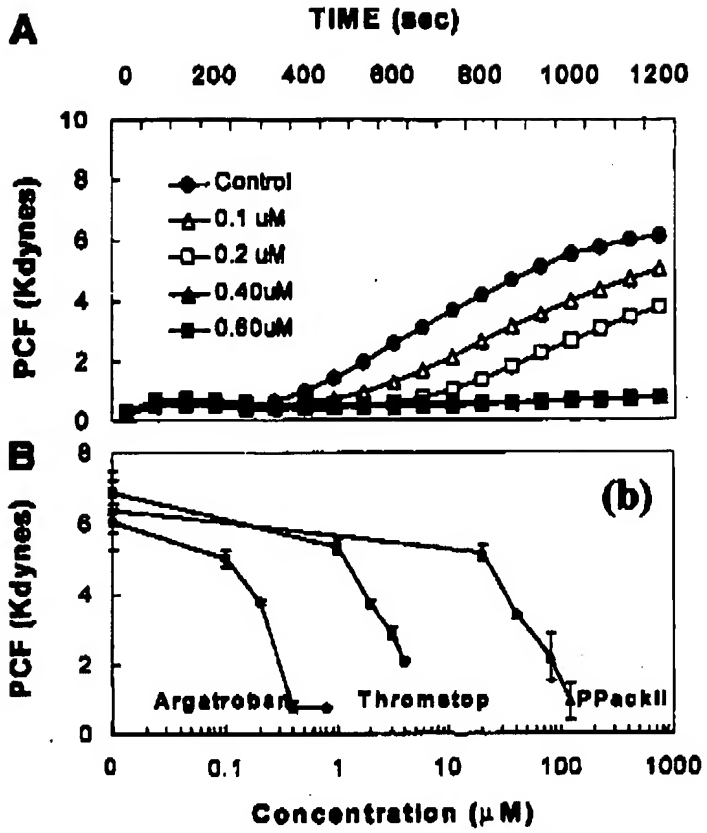

Fig. 3. A: Effect of increasing concentrations of argatroban on the kinetics of platelet contractile force (PCF) development in whole blood clotted by the addition of batroxobin (0.21 μg/mL) and calcium (10 mmol/L final concentration) at time zero. B: Effect of increasing argatroban (closed circles), thromstop (closed squares), and P-PACK II concentration (closed triangles) on platelet contractile force (PCF). Results are plotted as the mean ± SEM of duplicate measurements.

Table 2
Effect of Antithrombins on Absorbance (1200 s After Addition of Batroxobin and Calcium Chloride) and Fibrin Mass/Length Ratio

Parameter	Added inhibitor (μM)	Absorbance at 12000 s	Fibrin mass/length ratio (Daltons/cm)
Control	0	1.015	1.35
AT III	12	1.129	1.44
Thrombomodulin	0.04	1.122	1.34
Hirudin	0.30	1.032	1.24
Lepirudin	0.57	1.028	1.29
Bivalirudin	6.40	1.056	1.34
P-Pack II	120	1.015	1.23
Thromstop	4.0	0.994	1.24
Argatroban	0.2	1.136	1.31

recovery of PCF *(38)*. We recently demonstrated that the onset of PCF in whole blood occurs concurrently with the appearance of prothrombin activation fragment 1 + 2 in the system *(31)*.

Antithrombins, whether physiologic, hirudin-like, or synthetic, delay onset of PCF and reduce the values of PCF and CEM obtained after 20 min of clotting. Turbidity measurements reveal that, at the doses used in this study, these agents do not directly alter fibrin polymer structure. Therefore, the changes seen in CEM are predominantly the result of altered PCF. PCF increases CEM by putting the fibrin network under stress. Typically, stressed structures are more rigid. Thrombin concentration can alter fibrin structure by altering polymerization kinetics *(39,40)*. Higher thrombin activity causes faster fibrin formation and results in thinner fibrin fibers. Clots predominantly composed of thin fibers are more rigid. This effect did not come into play in this study since the fibrin network was already formed (by atroxin) before the generation of thrombin. A significant component of the effect of heparin on platelets is probably mediated by antithrombin effects.

Exogenous thrombin was not added in the measurements performed for this study. Therefore, the thrombin produced was the product of the intrinsic pathway activated upon the recalcification of the whole blood sample. Calcium was added at time zero, and the lag phase of approx 5 min is the amount of time required to produce thrombin adequate to support PCF development. This period is in reasonable agreement with the predictions of kinetic models based on the known biochemical properties of the enzymes involved in the system *(36)*, and also with results obtained in model plasma systems containing physiologic concentrations of plasma proteins and platelets *(37)*. The samples used in this study are whole blood and therefore contain platelets, monocytes, other white cells, and erythrocytes. Because the conversion of prothrombin to thrombin involves the formation of the prothrombinase complex on the platelet surface, the lag phase serves as a marker of platelet-supported thrombin production. The increase in lag phase before development of PCF noted with some of the antithrombins utilized in this study may indicate that certain antithrombins are capable of decreasing prothrombin conversion on the platelet surface. Because thrombin is the most profound known activator of platelet function, this implies that at least some antithrombins will have significant antiplatelet effects. The reverse may also be the case, with some antiplatelets exerting anticoagulant effects by decreasing or delaying prothrombin conversion on the platelet surface. Indeed, there may already be evidence for this with Abciximab *(41)*.

The ability of this modified PCF assay to simultaneously detect decreased platelet function, modified clot structure, and altered thrombin generation may offer simple ways to detect and monitor the global effects of a wide variety of anticoagulant and antiplatelet agents. This will become increasingly important as very potent combinations of antiplatelet, anticoagulant and fibrinolytic agents are used in patients with acute arterial events, such as myocardial infarction and stroke.

The PCF measurement may have potential as an assay of the therapeutic effects of direct thrombin inhibitors. The effects of these inhibitors are currently difficult to monitor *(42)*. As seen in Fig. 3, suppression of PCF can distinguish the relative potencies of serine proteases in whole blood. Argatroban is more potent than Thromstop, which is more potent than P-PACK II. Because the assay is dependent on endogenous thrombin production, the spectrum of serine protease inhibition can be used to identify the relative importance of various enzyme pathways in thrombin production. The fact that P-PACK II, at concentrations that should have totally suppressed the contact pathway, had little effect on PCF provides evidence that VIIa-platelet interactions are the predominant pathway for thrombin production in recalcified whole blood.

It may be possible to measure the global effects of any antithrombin (or any other type

of anticoagulant) on clot structure (CEM), platelet function (PCF), and thrombin generation (TGT) in real time (>30 min) on a single 700-μL sample of whole blood. Demonstration of the true potential of the technique will await ex vivo measurements in patients being treated with these agents.

Finally, previous investigations have emphasized the influence of factor deficiencies on the rate of thrombin generation and/or the rate of fibrin formation (25). The present study demonstrates that even when the fibrin structure is present before thrombin formation, thrombin generation still dramatically alters the structure through the advent of PCF formation. It is now apparent that although fibrin structure minimally affects PCF (43), PCF dramatically alters clot modulus.

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Attachment 2
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REVIEW ARTICLE

Development of Platelet Contractile Force as a Research and Clinical Measure of Platelet Function

*Marcus E. Carr, Jr.**

Coagulation Special Studies Laboratory, Division of Hematology/Oncology, Central Virginia Center for Coagulation Disorders, Departments of Medicine and Pathology, Medical College of Virginia, Virginia Commonwealth University, and Richmond Veterans Administration Medical Center Richmond, VA 23298

Abstract

This article reviews work performed at the Medical College of Virginia of Virginia Commonwealth University during the development of a whole-blood assay of platelet function. The new assay is capable of assessing platelet function during clotting and thus allows measurement of the contribution of platelets to thrombin generation. Because platelets are monitored in the presence of thrombin, the test gages platelets under conditions of maximal activation. Three parameters are simultaneously assessed on one 700-µL sample of citrated whole blood. Platelet contractile force (PCF), the force produced by platelets during clot retraction, is directly measured as a function of time. This parameter is sensitive to platelet number, platelet metabolic status, glycoprotein IIb/IIIa status, and the presence of antithrombin activities. Clot elastic modulus (CEM), also measured as a function of time, is sensitive to fibrinogen concentration, platelet concentration, the rate of thrombin generation, the flexibility of red cells, and the production of force by platelets. The third parameter, the thrombin generation time (TGT) is determined from the PCF kinetics curve. Because PCF is absolutely thrombin dependent (no thrombin—no force), the initial upswing in PCF occurs at the moment of thrombin production. TGT is sensitive to clotting factor deficiencies, clotting factor inhibitors, and the presence of antithrombins, all of which prolong the TGT and are known to be hemophilic states. Treatment of hemophilic states with hemostatic agents shortens the TGT toward normal. TGT has been demonstrated to be shorter and PCF to be increased in coronary artery disease, diabetes mellitus, and several other thrombophilic states. Treatment of thrombophilic states with a variety of heparin and nonheparin anticoagulants prolongs the TGT toward normal. The combination of PCF, CEM, and TGT measured on the same sample may allow rapid assessment of global hemostasis and the response to a variety of procoagulant and anticoagulant medications.

Index Entries: Clot retraction; platelet contractile force; elastic modulus; thrombin generation; thrombophilia; hemophilia.

* Author to whom all correspondence and reprint requests should be addresed. E-mail: mcarr@hsc.vcu.edu

INTRODUCTION

The article describes work performed in an effort to develop a simple and quick assay of platelet function that would correlate with bleeding risk. For the past 50 yr, such assays have been available for testing fluid-phase coagulation. The prothrombin time (PT) is sensitive to deficiencies in or inhibition of the tissue factor (extrinsic) pathway *(1)*. In like manner, the partial thromboplastin time (PTT) allows assessment of the contact (intrinsic) pathway *(2,3)*. These assays are simple, inexpensive, quick, and widely available. More importantly, they provide information that is relatively easy to interpret and that correlates with bleeding risk. Because these assays are typically performed in platelet-poor plasma, neither provides useful information regarding platelet function.

Until recently, the most useful platelet test has been the platelet count. If the platelet concentration falls below 20,000/μL (a quantitative platelet abnormality), the risk of spontaneous bleeding increases dramatically *(4)*. Unfortunately, there are times when the platelet count is normal, but the platelets themselves are inherently (qualitatively) dysfunctional. Identification of the later state continues to be problematic.

For many years, the primary test of platelet function has been the bleeding time *(5–7)*. The bleeding time is performed by making a standard skin incision and measuring the time required for the bleeding to stop. While intuitively pleasing, the bleeding time assay is remarkably operator dependent *(8,9)*, and studies of its reproducibility and predictive value are limited. Koster et al. found the standard deviation for the ratio of duplicate runs to vary from 0.33 to 0.37, depending on the technique *(10)*. Schwartz et al. found the coefficient of variation in normal volunteers to be 18% for duplicate runs done by the same technician *(11)*. The coefficient of variation was closer to 30% if the test was performed 6 wk after the initial determination. An analysis by Rodgers and Levin of literature published prior to 1990 found that when data were adequate for statistical assessment, the characteristics of the bleeding time were statistically indistinguishable from those of a "completely noninformative test" *(8)*.

Platelet aggregations have been utilized since the early 1960s to evaluate conditions related to abnormal platelet receptor function, platelet activation, and platelet secretion *(12,13)*. By utilizing a variety of platelet activators (agonists), the competence of glycoproteins Ib and IIb/IIIa, the ability of platelets to produce thromboxane, and the ability of platelets to secrete granular contents can be measured. Thus, platelet aggregation studies contribute to the evaluation of Bernard–Soulier disease (glycoprotein Ib), Glanzmann's thrombasthenia (glycoprotein IIb/IIIa), storage pool diseases, and aspirin effects. Because of the ability of ristocetin to induce platelet agglutination in the presence of von Willebrand factor, platelet aggregations also have utility in the evaluation of von Willebrand disease.

Unfortunately, platelet aggregation studies are time-consuming and technically demanding and the results are open to interpretation. Because they are performed on anticoagulated blood, aggregation studies cannot be used to test platelets under conditions of maximal activation, and they are incapable of assessing the role of platelets in thrombin production. In addition, platelet aggregations tend to be sensitive to relatively mild abnormalities of platelet function and are not predictive of bleeding risk. The classic example of both the sensitivity and lack of correlation of platelet aggregation with bleeding is seen with aspirin. One aspirin will dramatically alter platelet aggregation, but the patient has virtually no increased bleeding risk.

The need for a better test of platelet function has increased in recent years with the introduction of a variety of potent anticoagulant and antiplatelet medications. These medications are designed to block platelet function in clinical situations known to be associated with significant risk of arterial thrombosis. Two important applications are the treatment of patients with transient ischemic attack (TIA),

the prelude to stroke, and unstable angina, the harbinger of myocardial infarction. The primary side effect of these medications is increased bleeding risk. Assays that are sensitive to the therapeutic effects of medications and predictive of excessive bleeding risk would be of clinical benefit. Although modified forms of platelet aggregation *(14–16)* and assays sensitive to both adhesion and aggregation *(17,18)* have been introduced, neither can be performed in the presence of thrombin. Thus, the ability to study the contribution of platelets to clot formation or to study platelet function under conditions of maximal activation has not been available.

ASSESSMENT OF CLOT RETRACTION

Although most platelet function assays, including aggregation, adhesion, and secretion studies, assess platelet function in the absence of clotting, clot retraction measures a platelet-dependent parameter that only occurs during clot formation. Employed for more than 200 yr, clot retraction first came into vogue during the time of medical blood letting. During blood letting, blood was collected in a basin where it would clot. The red gelatinous mass was turned over onto a piece of cheesecloth. During clot retraction, expressed serum was trapped in the cheesecloth. The remaining material was termed the caressmentum. If the amount of caressmentum was small, blood letting had to be decreased. Thus, in its early use, clot retraction served as a crude hematocrit. Early investigators noted that clot retraction was dependent on the presence of small corpuscles, subsequently termed "platelets." Glanzmann identified the absence of clot retraction as a classic finding in the profound bleeding disorder termed "thrombasthenia" *(19)*. Thrombasthenia actually means "weak platelets." Subsequently, clot retraction was studied by a variety of techniques in a wide spectrum of disease states. Much of this literature was summarized in 1949 in text on the subject by Budtz-Olsen *(20)*. Techniques for assessing clot retraction have typically measured the volume of extruded serum or estimated the decrease in the size of the shrunken clot mass. Although the physiologic purpose of clot retraction has never been fully explained, the fact that 34% of the dry weight of a platelet is the contractile protein actin speaks to the potential importance of this process.

More recent studies of clot retraction have employed electron microscopy to evaluate platelet–fibrin interactions during clotting. Early in the process, small platelet aggregates can be seen. These aggregates increase in size and are clearly associated with fibrin strands. The strands adhere tightly to the platelets and actually form grooves within the platelet surface. As clotting proceeds, the platelets extend pseudopodia along fibrin fibers. The pseudopodia adhere to the fibrin, utilizing the same glycoprotein IIb/IIIa receptor used to bind fibrinogen during platelet aggregation. The formation of pseudopodia is an energy-dependent event and requires shifts of intracellular calcium. Once the pseudopodia are extended along the fibrin, they are pulled back toward the central mass of the platelet. The combined effect of thousands of pulling platelets causes early alignment of fibrin fibers and the production of stress throughout the network. Putting the network under stress dramatically increases its structural integrity, leading to increased clot elastic modulus. The process begins early in the clotting process, virtually as soon as thrombin is produced. Some have postulated that the process is designed to pull the wound edges into approximation. Another potential consequence would be early re-establishment of blood flow in the injured blood vessel. Via clot retraction, platelets may rapidly re-establish the vessel lumen by pulling the clot mass out of the bloodstream and concentrating it against the injured vessel wall.

Current clinical assays of clot retraction measure clot shrinkage after a set period of time. Such measurements are affected by several clot properties that are relatively independent of platelet function. These properties include the adhesive forces that anchor the clot to a containment vessel and the structural

resistance of the clot to deformation. Strong adhesion of the clot to the containment vessel and/or a high clot elastic modulus will delay the apparent onset and reduce the extent of clot retraction independent of the forces generated by the platelets. Scanning electron micrographs reveal platelet-induced structural alterations within the first 90 s of clotting. The early onsets of force development and clot retraction are not appreciated with earlier measurement techniques that rely on clot shrinkage.

DEVELOPMENT OF THE DEVICE AND TECHNIQUE FOR MEASURING PLATELET CONTRACTILE FORCE

The techniques developed at the Medical College of Virginia permit direct measurement of the forces produced in the sample during clot formation. The initial prototype was made of plastic Petri dishes. The sample was loaded into the shallow-bottom dish and a flat upper plate was lowered onto the upper surface of the forming clot. The upper disk was attached to a strain-gage transducer that generated a voltage in proportion to the amount of stress. As the clot formed and the platelets began to pull within the network, a downward force was transmitted to the upper plate and transducer. As the downward force stressed the transducer, a voltage proportional to the distance moved was generated. Because the transducer actually measured the distance moved, a calibration constant correlating distance moved to force applied was established. Initially the calibration constant was calculated only after the force curve had reached a plateau. A weight of mass (M) was placed on the upper surface of the transducer and the voltage deflection because of the weight was measured. The downward force produced by the weight was

$$F = Mg$$

where g is the gravitational constant. Using this simple technique, the magnitude of the applied force was well defined and the calibration constant was easily derived. Because the upper plate did not completely cover the clot surface, a thin coating of oil was applied to the exposed edge to prevent evaporation. Early work with this device confirmed that the forces produced by platelets in platelet-rich plasma or whole-blood clots were significant (several kilodynes in magnitude) and easily measured (*21*). The onset of force development occurred as soon as the fibrin network was in place. The results were consistent with work preformed by other researchers working with preformed strips of clot mounted in a device used to measure muscle function (*22*).

A significant degree of daily variability complicated work performed with the plastic prototype. Subsequent studies revealed that a significant component of the variability was the result of the temperature dependence of the reactions. Platelet force development in clots formed at room temperature (27°C) was much less than that seen at 37°C. Force development was totally suppressed at and below 15°C (*23*). The first prototype was also relatively difficult to align and the distance (gap) between the upper plate and cup tended to vary. These deficiencies were corrected through the fabrication of a series of aluminum and stainless-steel prototypes that incorporated temperature control (via a water jacket and thermostated water bath), a rigid coupling arm between the transducer and upper plate, and various alignment and gap setting techniques (Fig. 1A). Utilizing the newer devices, platelet force development was found to be directly dependent on platelet count, sensitive to temperature, sensitive to calcium concentration, but relatively independent of fibrinogen concentration over the normal fibrinogen range of 100–400 g/dL (*19*). Cytochalasin B produced dose-dependent inhibition of force, confirming the importance of actin polymerization. At 10 μg/mL, cytochalasin B reduced by 70% the force produced after 1200 s of clotting. Disruption of microtubules by vincristine (0.2–0.6 mM) and colchicine (2.5–20 mM) produced dose-dependent decreases in force, but vinblastine at concentrations as high as 40 mM had little effect. The importance of cAMP levels was confirmed

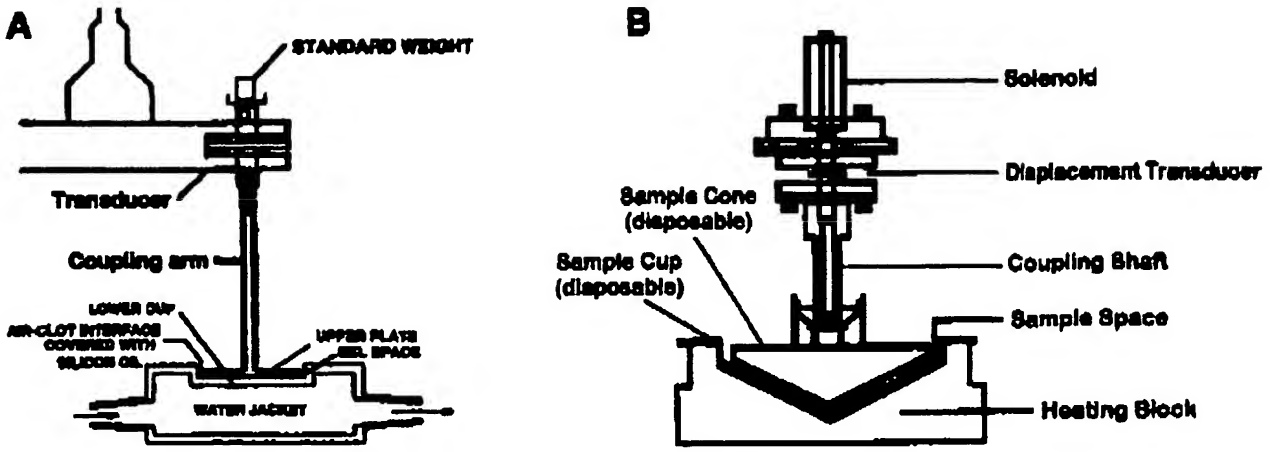

Fig. 1. (A) Schematic diagram of second prototype instrument that utilized a water jacket to solve the temperature problem and allowed repetitive measurement of clot elastic modulus via direct imposition of a downward force on the transducer. (B) Schematic diagram of the RM2 instrument that utilized a cone–cone geometry to circumvent the problem of sample evaporation. The sample volume was also significantly reduced from 1700 to 700 μL.

by demonstration of force inhibition by both prostaglandin E_1 PGE_1 and DBcAMP. At a concentration of 10 μ*M*, PGE_1 decreased the force to 20% of baseline. Thirty micromolars of DBcAMP reduced the force by 50%, and at 300 μ*M* the force was completely inhibited.

Demonstration of the sensitivity of platelet contractile force (PCF) to the effects of inhibitors of platelet function raised the possibility that PCF might prove useful in the evaluation of patients at increased risk for bleeding because of platelet dysfunction. The first opportunity to test the new technique occurred on the second day of thermostated prototype testing. A 34-yr-old male developed acute renal failure as a result of severe uncontrolled hypertension *(24)*. He subsequently began to bleed from his stomach. After a 10-unit transfusion of packed red cells, a partial gastrectomy appeared to stop the bleeding. Unfortunately, 4 h postprocedure he rebled 8 additional units of blood and was transferred to Medical College of Virginia/Virginia Commonwealth University (MCV/VCU) for additional treatment. His hemorrhage was obviously worsened by the development of uremic platelet syndrome. This qualitative platelet disorder develops in the setting of uremia, decreases platelet function, and markedly increases the risk of bleeding. Several potential treatments with established clinical benefit are available, but laboratory documentation of their effects has been difficult. Shortening of the bleeding time is typically used to document improvement, but the deficiencies of this test have already been discussed. In this patient, blood samples were tested before and after treatment with *d*-arginine vasopressin (DDAVP). DDAVP causes prompt release of von Willebrand factor from endothelial cells and is the agent of choice in the treatment of uremic platelet dysfunction. PCF measurements confirmed markedly decreased force development prior to DDAVP (*see* Fig. 2A) and remarkably enhanced function after drug administration (*see* Fig. 2B). Platelet aggregation studies also improved, but the bleeding time was unchanged. Most impressively, improved PCF development was accompanied by cessation of bleeding. The institution of dialysis and administration of erythropoietin also improve uremic platelet syndrome. In chronic dialysis patients, the risk of bleeding is frequently replaced by increased risk of shunt thrombosis. Subsequent studies in chronic hemodialysis

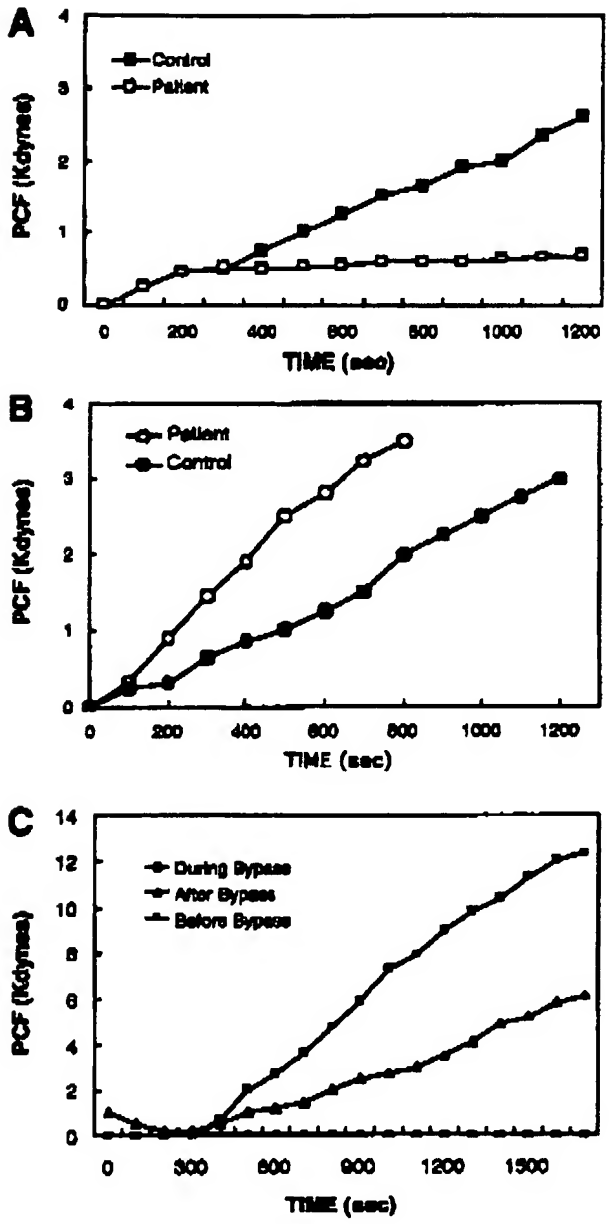

Fig. 2. (A) Platelet force development in a gel formed from blood uremic patient obtained prior to administration of DDAVP. Force is plotted in thousand dyne units and represents the total force exerted on the upper plate. Time in seconds is plotted on the *x*-axis with time 0 being the moment of thrombin and calcium addition. (B) Platelet force development in a gel formed from blood obtained 2 h postadministration of DDAVP (0.3 μg/kg). The control donor was not exposed to DDAVP. (C) Effect of cardiopulmonary bypass (CPB) on PCF in blood taken from patients before CPB, during CPB on high-dose heparin, and after reversal of heparin by protamine sulfate. All blood was clotted by the addition of batroxobin (0.1 μg/mL) and calcium (10 m*M*) at time 0. PCF is totally suppressed in the presence of high-dose heparin, but it recovers significantly after the administration of protamine sulfate.

patients revealed normal to increased force development in this population (25).

DEVELOPMENT OF THE TECHNIQUE FOR MEASURING CLOT ELASTIC MODULUS

Further development of the early device centered on appropriate means of calibration. As noted previously, the displacement transducer measures distance moved rather than force. The initial solution was to measure a calibration constant at the end of the force kinetic measurement. The calibration number was then used to convert each voltage to the appropriate force. Unfortunately, this technique overestimated the magnitude of the forces developed early in the reaction. The overestimation occurred because the calibration constant measured at the end of clot formation was only appropriate for the clot structure present at that time. Early in the clotting process, no gel structure was present. A force calibration constant (dynes per volt) measured during this phase would have been significantly smaller than one determined after a solid clot (which will offer much more resistance to deformation) had formed. Thus, to truly assess the kinetics of force development, the calibration constant had to be repeatedly measured. Although this initially appeared to be a nuisance, it was soon appreciated that the force calibration constant was actually a measurement of clot elastic modulus

(CEM). This parameter, although related to PCF, provides additional information about clot structure and integrity *(26)*. CEM increases with PCF, but, unlike PCF, CEM is directly dependent on fibrinogen concentration *(27)*. CEM is also significantly influenced by red cell flexibility. This is easily demonstrated by the effects of irreversibly sickled erythrocytes. If one increases the concentration of such cells in blood samples, the elastic modulus of clots formed from these samples dramatically increases *(28)*.

The potential clinical utility of CEM was initially noted in patients with bleeding as a result of enhanced fibrinolysis. The initial case was a 41-yr-old male who came to the emergency room complaining of pain in his right leg that had gradually increased over the previous 7 d *(29)*. There was no history of trauma or overexertion. He was discharged to home with a prescription for acetaminophen and instructions to return if the pain did not resolve. He returned to the hospital 4 d later with his right leg purple from his ankle to the mid-thigh and a new complaint of dizziness. Physical examination revealed a swollen left leg, but neurological examination was normal and pulses were palpable. Dizziness was the result of orthostatic hypotension and anemia. His hemoglobin, which had been normal on his initial visit, had fallen to 8.8 g/dL. Laboratory parameters revealed a normal white count, mild thrombocytopenia (platelet count 133,000/μL), normal prothrombin time, normal partial thromboplastin time, normal creatinine phosphokinase, and an elevated fibrinogen level (475 mg/dL). All cultures and toxin screens were negative. Concern was raised regarding impending vascular and neurologic compromise in the lower leg. If the increased tissue pressures are not relieved, this process (termed "compartment syndrome") can rapidly lead to muscle necrosis, myoglobinemia, renal failure, and either amputation of the involved extremity or patient death. Computed tomography (CT) and magnetic resonance imaging (MRI) scans failed to identify the cause of the bleeding. A hematology consult was obtained regarding

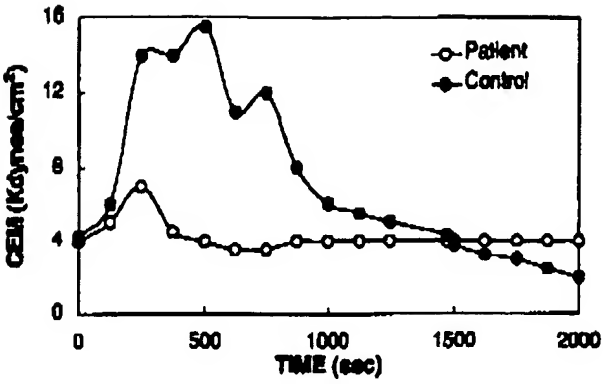

Fig. 3. Abnormal clot formation and dissolution in patient's blood clotted with thrombin and dissolved with tissue plasminogen activator. Both agents were added at time 0. The addition of identical amounts of these agents to normal blood produced the control curve.

the source of the problem and recommendations for control of the hemorrhage. Additional evaluations revealed a normal bleeding time, normal platelet aggregations, and normal platelet contractile force. Clot elastic modulus revealed a deficient clot structure, which rapidly dissolved in the presence of a small amount of tissue plasminogen activator (*see* Fig. 3). Treatment of his hyperfibrinolysis was instituted with ε-aminocaproic acid (EACA) and correction of rapid lysis was documented with repeat CEM measurements. Within 4 d, his situation had improved to the point that he could be released from the hospital. The ability to detect abnormal fibrinolysis in whole blood and to document correction of the problem with antifibrinolytics led to the development of a new assay termed the "tPA-induced clot lysis time" (TCLOT). This has proven to be useful in the identification of abnormal fibrinolysis in both pediatric and adult patients *(30)*.

DEVELOPMENT OF THE FIRST PCF RESEARCH SYSTEM

Although the first generation of prototypes allowed assessment of research potential, they

were cumbersome to set up and they lacked any real potential for the increased sample throughput necessary to support clinical testing. Based on the original geometry, an instrument was designed that would facilitate sample loading, geometric alignment, temperature control, and signal acquisition. This initial unit, termed the "Research Model 1" (RM1), interfaced with a computer and software package whose output included time-course displays of the raw data signal, the platelet contractile force curve, and the clot elastic modulus curve *(31)*.

Reproducibility was sufficient to allow initiation of several small clinical trials that assessed the utility of the PCF and CEM parameters in the detection of platelet abnormalities. Because the geometry was unchanged, the RM1 continued to have an exposed upper sample surface and to require the application of inert oil to prevent evaporation.

Initial Clinical Studies: Assessment of Platelet Function During Cardiopulmonary Bypass Grafting

Platelet function is altered during cardiopulmonary bypass grafting (CPB), and the resulting deficient function is thought to be a major determinant of the amount of blood lost *(32–34)*. A pilot, 10-patient trial was initiated to assess changes in PCF during CPB *(35)*. Because high-dose heparin (4 U/mL) is routinely used during CPB, clotting could not be initiated by addition of thrombin. However, clots could be formed by the addition of batroxobin and calcium. Batroxobin, a snake venom enzyme, directly cleaves the A-peptide from fibrinogen but is not inhibited by antithrombin III. As a consequence, batroxobin causes rapid fibrin formation even in the presence of large amounts of heparin. Because batroxobin does not activate platelets, there is a lag phase prior to the onset of PCF in the batroxobin system. During this time period, thrombin is generated by recalcification. Because the fibrin network is in place prior to the generation of thrombin, PCF becomes apparent as soon as a small

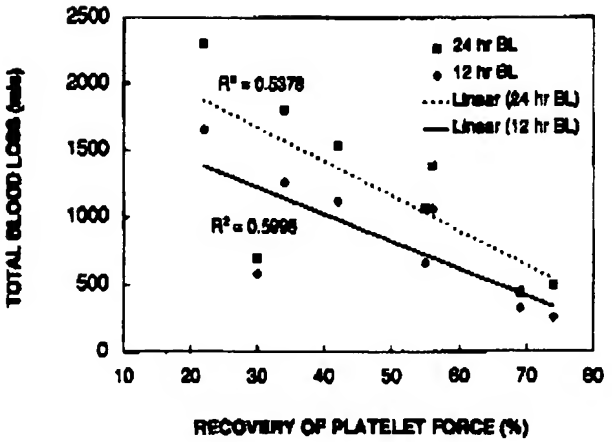

Fig. 4. Correlation of the extent of PCF recovery after protamine sulfate with blood loss at 12 and 24 h in eight patients undergoing CPB.

amount of thrombin is generated. Blood taken from patients prior to CPB demonstrated brisk PCF development when clotted with batroxobin and calcium (*see* Fig. 2C). During CPB, PCF was totally suppressed. After heparin reversal with protamine sulfate, PCF returned but never reached its preoperative levels. The degree to which PCF recovered varied among patients. When percent recovery of the PCF was plotted against blood loss during the first 12 and 24 h of the perioperative period, a surprisingly strong inverse correlation was noted (*see* Fig. 4). Patients with a good return of PCF had less blood loss. Lack of complete return of PCF undoubtedly results from a combination of altered platelet membrane and partial degranulation thought to occur as a result of exposure of the platelets to the extracorporeal circuit *(34,36–38)*. Incomplete reversal of heparin by protamine sulfate may also play an important role and could be a significant component of the variability in PCF recovery seen in different patients. Subsequent in vitro investigations revealed that protamine sulfate reversal of high-level heparinization could result in significant ongoing platelet suppression even when heparin anticoagulant effects appear to be completely reversed *(39)*.

Although the results in CPB were encouraging, an after-action design review revealed several significant problems with the RM1. First, RM1 was expensive to make and expensive to repair. Second, the sample size of 1.7 mL of blood was excessive for pediatric patients. Third, the requirement for a covering oil to prevent evaporation was seen as a significant limiting factor for clinical use. These problems were addressed during the development of the RM2, the first instrument produced in significant quantities. A change in geometry from flat–flat to cone–cone surfaces and a reduction in sample volume to 700 µL produced a situation in which the sample edge was trapped between two surfaces (*see* Fig. 1B). The new geometry shifted the evaporation force vector, making it more perpendicular than parallel to the measurement force vector. The reduced vapor exchange as a result of sample trapping and the reduced impact of evaporation on the force signal eliminated the need for a covering oil. Additional electrical and mechanical design changes placed a computer in the instrument and provided for two-way communication between the operator and the instrument. The design changes reduced the number of machined parts by a factor of 10, made maximal use of off-the-shelf electronics, and reduced instrument cost significantly. Production of the RM2 has allowed the evaluation of instruments at multiple sites and the definition of normal values for PCF and CEM.

RM2: Normal Values and Reproducibility

Work done at several sites has yielded comparable normal ranges for asymptomatic volunteers. At MCV/VCU, PCF values for females (7.41 ± 0.29 kdyn) were slightly higher than for males (6.45 ± 0.29 kdyn). Similar results were seen in Denver, Co (females: 6.92 ± 0.23 kdyn; males: 5.88 ± 0.28 kdyn), confirming the Richmond results and demonstrating the limited effects of altitude *(40)*.

Reproducibility of PCF and CEM measurements at three sites (Virginia, Colorado, and Texas) in the United States and two sites in Europe (Germany and Italy) was between 2% and 17% CV (coefficient of variation) for either parameter. The CV for PCF ranged from 2% to 16% for all observers at all sites, whereas the CV for CEM ranged from 5% to 17%. Female operators appeared to have lower CVs and there appeared to be some degree of learning in that the CV tended to fall over time for the same observer. The CV for PCF for most technicians at each study site was < 10% and is significantly less than the quoted CVs for the bleeding time. The higher CV for CEM has been determined to be at least partially because of the efficiency of pipetting and sample mixing. These deficiencies are being addressed by modification of the RM2 to include an automated mixing step.

As noted previously for platelet aggregation studies, the platelet function is stable at room temperature for a minimum of 4–6 h. When compared to aggregation studies however, PCF is a much hardier parameter. Samples of whole blood were held at room temperature for up to 16 d. Each day, the samples were assayed for platelet aggregations and PCF. Platelet aggregations were dramatically decreased at 24 h and completely absent at 48 h. PCF persisted in all samples for at least 10 d, and in two samples, PCF was detectable far beyond what is reported to be the total life-span of platelets. This finding was unexpected and may indicate that the life-span of platelet outside of the body is longer than 10 d. The mechanism of normal platelet senescence within the body is incompletely known. Even the final place of platelet removal has not been firmly established. We have clearly shown that PCF is absent when platelets are not in the sample. The finding of prolonged extracorporeal platelet activity may mean that once they are removed from the body, platelets can be held for significantly longer periods than the current limit of 4 d. Prolonged platelet storage would require adequate suppression of bacterial contamination and growth, but could significantly expand the available supply of platelets without requiring additional donors. It should be

noted that the platelets in this study were held in whole blood instead of the platelet-rich concentrates normally harvested for transfusion. This may indicate platelet-protective properties of erythrocytes and leukocytes that have previously not been considered.

When individual normal volunteers were repeatedly assayed over a 12-mo period, changes in PCF were negligible. The robust nature of the parameter and its absolute dependence on platelet viability have led some groups to examine the use of the PCF parameter as a marker of platelet survival in stored and modified platelet preparations *(41,42)*.

Two critical assay parameters for the present PCF technique are the concentration of platelets and the surface area over which the measurement is made (i.e., the area in contact with the transducer surface). The platelet count is typically known from concurrent blood cell analysis, and the later is taken into consideration in the mathematical analysis. The PCF measurements made at a variety of sites, by a number of different technicians, utilizing a variety of prototype instruments have been reassuringly consistent.

DEVELOPMENT OF THE THROMBIN GENERATION TIME AS A COMPONENT OF THE PCF ASSAY

As mentioned previously, citrated blood clotted with batroxobin and calcium develops PCF. Because batroxobin does not activate platelets, there is a lag phase prior to the onset of PCF in the batroxobin system. During this time period, thrombin is generated by recalcification. Because the fibrin network is in place prior to the generation of thrombin, PCF becomes apparent as soon as a small amount of thrombin is generated (*see* Fig. 5A). Thus, the inflection or takeoff point in the PCF curve serves as a marker of thrombin generation in the batroxobin-mediated assay. In normal individuals, PCF developed by the addition of batroxobin differs only in the time of onset. However, if thrombin generation is inhibited by the addition of anticoagulants or by the presence of clotting factor deficiencies, PCF in the batroxobin clots is dramatically delayed and deficient. We designate the time between calcium addition and initial PCF development as the thrombin generation time (TGT). Similar results can be obtained without using a snake venom enzyme if one simply recalcifies the plasma sample. Because thrombin generation must be followed by fibrin clot formation before PCF can begin to occur in the recalcification assay, the lag phase tends to be longer. Similar assays termed "reptilase clot retractions" were shown in the early 1970s to be sensitive to a variety of antiplatelet and anticoagulant agents *(43)*. TGT is sensitive to the effects of heparin *(44,45)*, low-molecular-weight heparins *(46)*, dermatan sulfate *(47)*, nonheparin antithrombins *(48)*, inherited clotting factor deficiencies *(49)*, and clotting factor deficiencies induced by warfarin. In vitro studies indicate the potential for documentation of the correction of deficient thrombin generation by hemostatic agents such as recombinant FVIIa (*see* Fig. 5B).

RECORDING AND REPORTING PCF, CEM, AND TGT RESULTS

In most early studies, the reported PCF value was one obtained after an arbitrary period of time. Typically, the assay ran for 1200 s. PCF in clots initiated by direct addition of thrombin and calcium approached a plateau value during this period. The same was true in the majority of "normal" samples clotted with batroxobin and calcium. The recognition that the initial upswing in PCF in the batroxobin assay could serve as a marker of thrombin generation provided impetus for reporting the kinetics of PCF and CEM development. The current system displays the kinetic curves as they develop and provides an electronic and a printed report of the kinetic plots and the numerical data points. The TGT is determined from the kinetic plot as the time elapsed before the onset of rapid force development. The assay time remains an

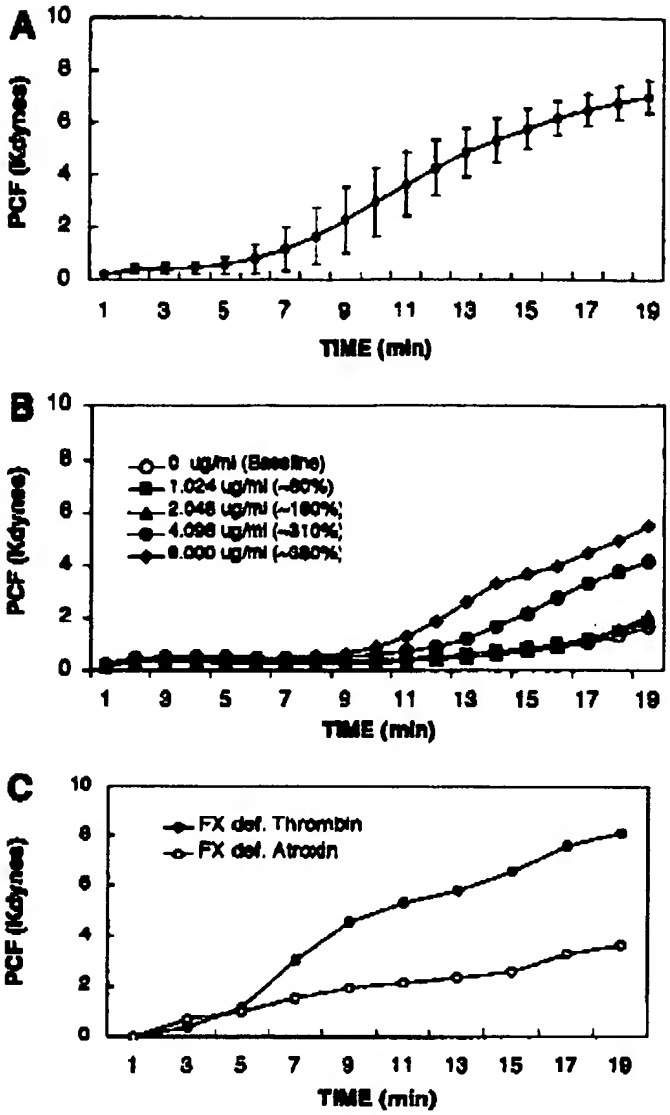

Fig. 5. Effects of clotting factor deficiencies on thrombin generation time (TGT) and PCF. (**A**) TGT and PCF development measured in whole blood from 20 normal controls clotted by the addition of batroxobin (0.1 μg/mL) and calcium (10 m*M*) at time 0. TGT time is determined by measurement of the lag phase prior to PCF development and is typically about 5 min. PCF is typically about 6500 dyn after 20 min. (**B**) Effect on TGT and PCF of adding increasing amounts of recombinant factor VIIa to whole blood from a patient with coumadin-induced multifactor deficiencies. Recombinant VIIa was added to the samples 1 min prior to the addition of batroxobin and calcium. (**C**) PCF development in platelet-rich plasma from a patient with factor X deficiency. Open circles are data from a clot formed by the addition of thrombin (1 NIH U/mL) and calcium (10 m*M*). Filled circles are data obtained from a clot formed by the addition of batroxobin (0.1 μg/mL) and calcium (10 m*M*). PCF is normal with thrombin addition but is delayed and reduced when clotting is initiated with batroxobin.

operator-set parameter and can be extended to greater than 1 h if so desired.

DISEASES ASSOCIATED WITH DECREASED PLATELET CONTRACTILE FORCE

Glanzmann's thrombasthenia (GT) is a significant bleeding disorder associated with abnormal platelet glycoprotein IIb/IIIa (GP IIb/IIIa). Because GP IIb/IIIa is the primary receptor for fibrinogen, the hallmark of GT is reduced or absent platelet aggregation in response to all agonists. Because GP IIb/IIIa is also the primary receptor by which platelets adhere to fibrin and through which they transmit their force to the network, GT is also associated with decreased or absent clot retraction. Although the incidence of GT is rather small, we have had the opportunity to study four such patients. In all cases studied to date, PCF has been dramatically reduced (*see* Fig. 6A).

As mentioned earlier, clotting factor deficiencies delay thrombin generation in clots induced by batroxobin and/or recalcification. As a consequence, PCF is delayed and reduced in such clots (*see* Fig. 5C). The degree to which various clotting factor deficiencies affect PCF differs. Deficiencies of factors VII, VIII, and XI reduced PCF, deficiency of IX had variable effects, deficiencies of X and V had minimal effects, and deficiencies of fibrinogen and XIII affected PCF in both thrombin and batroxobin assays *(49)*. The effects of factor VIII inhibitors are virtually indistinguishable from factor VIII deficiency in terms of their reductions of PCF.

As demonstrated earlier in the first case report (*see* Fig. 2A), acute uremia is associated with decreased PCF. In 20 patients with end-stage renal disease on chronic hemodialysis, only 1 patient had significantly reduced PCF. The patient with decreased PCF did suffer from increased hemorrhagic tendencies. In the other 19 patients, bleeding times were normal, PCF values were mildly elevated, and bleeding was not thought to be excessive *(25)*. The relative influences of dialysis and erythropoietin on these results could not be assessed in this limited trial. Similar results have been reported by another group *(50)*.

DISEASES ASSOCIATED WITH INCREASED PLATELET CONTRACTILE FORCE

Initial interest in PCF was as a potential marker of decreased platelet function and increased hemorrhagic risk. The first indication that patients might exhibit increased PCF in diseases associated with increased thrombotic risk was noted in the first cardiopulmonary bypass study. Patients with known coronary artery disease (CAD) who were undergoing coronary artery grafting were noted to have baseline PCF values that were more than double those of normal controls *(35)*. This occurred despite the fact that all these patients were on aspirin and some were on additional medications with known antiplatelet activity. Three patients with known CAD and contraindications to aspirin were subsequently found to have extremely high PCF values *(51)*.

More recently, a study of 100 patients presenting to the emergency department with chest pain found significant elevations in PCF in these patients vs normal controls *(52)*. Patients subsequently documented to have CAD by angiography, occurrence of a myocardial infarction, or the need for emergent revascularization had even higher PCF values (*see* Fig. 6B). The highest PCF values were found in diabetic patients presenting with chest pain (*see* Fig. 6C) *(53)*. Once again, most of the patients were on multiple antiplatelet agents. This was confirmed by documentation of significantly diminished platelet aggregation in the patients versus controls.

Thromboangiitis obliterans, Buerger's disease, is a nonatherosclerotic obliterative disease of large- and moderate-size arteries. It is associated with cigarette abuse and frequently leads to amputation of distal aspects of the hands and feet. We have studied several patients with this disease and have found very

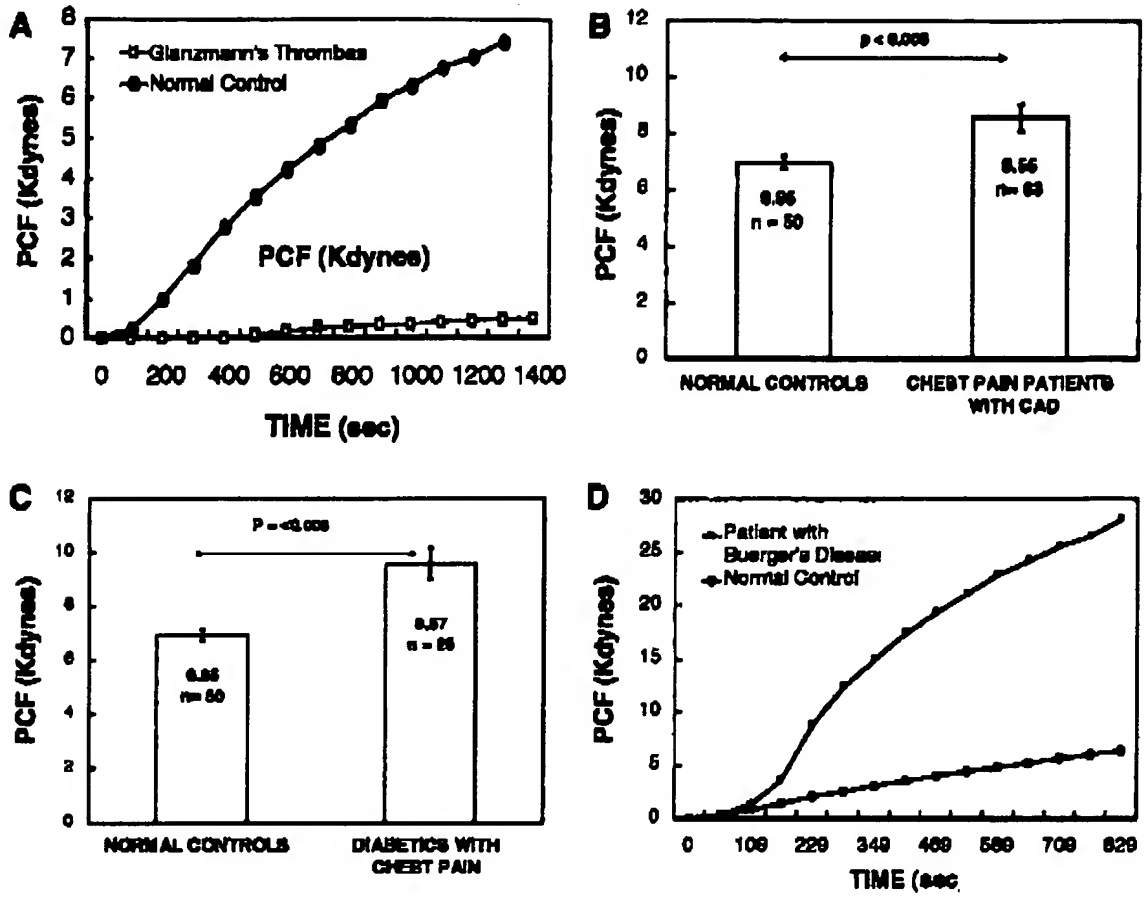

Fig. 6. (A) PCF development in a symptomatic patient with Glanzmann's thrombasthenia. Her asymptomatic parents served as controls. PCF was measured in platelet-rich plasma clotted by the addition of thrombin (1 NIH U/mL) and calcium (10 mM) added at time 0. (B) Patients with coronary artery disease presenting to the emergency department with chest pain have significantly elevated PCF. (C) PCF is significantly elevated in patients with diabetes mellitus presenting to the emergency department with chest pain. (D) Abnormally elevated PCF in a patient with Buerger's disease. Clots were formed by the addition of thrombin and calcium to platelet-rich plasma at time 0.

high PCF values (*see* Fig. 6D) *(54)*. Once again, all three patients were on aspirin.

DISEASES ASSOCIATED WITH ABNORMAL CLOT ELASTIC MODULUS

Multiple Myeloma

For 50 yr, abnormal clot retraction in multiple myeloma was felt to represent abnormal platelet function. In a study of 10 patients with myeloma, we found that PCF was normal or slightly elevated *(55)*. The abnormal clot retraction in this disease was the result of an altered fibrin structure. The myeloma protein affects fibrin polymerization resulting in much thinner fibrin fibers with dramatically reduced fiber diameter. Two consequences of this altered structure are decreased gel turbidity of the plasma clots and dramatically increased CEM. The clots were simply so rigid that they

could not be collapsed by the normal forces produced by the platelets within the clot. This type of analysis could not be performed without the ability to simultaneously assess PCF and CEM.

Diabetes Mellitus

Diabetes is a recognized hypercoagulable state *(56)*, and diabetics typically suffer a thrombotic death. CEM is elevated in diabetes *(53)*. There are at least two causes. First, fibrinogen levels are elevated in these patients *(57–59)*. Second, the fibrinogen of diabetics becomes hyperglycosylated. This occurs because fibrinogen is a glycoprotein and has a very long plasma half-life. Glycoproteins will continue to add glucose if they are exposed to high glucose levels. When hyperglycosylated fibrinogen is clotted, it forms abnormal structures that are composed of thin fibrin fibers *(60)*. As with multiple myeloma, these clots are more rigid and more resistant to fibrinolysis *(61)*. Although inhibition of fibrinolysis should increase the thrombotic risk, it is unclear at this point whether changes in CEM are reflective of increased risk.

Hyperfibrinolysis

Assessment of fibrinolysis has been complicated by a lack of a simple, screening test. The TCLOT assay described earlier may be a reasonable starting point for test development. Using this assay, we have evaluated a series of adult and pediatric patients with bleeding tendencies whose only identifiable abnormality was enhanced fibrinolysis *(30)*. We demonstrated in vitro correction of the abnormality by the direct addition of EACA in their blood samples *(62)*. In addition, laboratory documentation of the response to EACA administration could be documented *(30)*. Although the TCLOT assay can be performed optically on plasma, the mechanical TCLOT performed by monitoring CEM offers the advantage of a whole-blood system and reflects true changes in structural analysis that can be missed when monitoring optical density *(29)*.

EFFECTS OF MEDICATIONS ON PCF: ANTICOAGULANTS

Heparin

As mentioned earlier, inhibitors of prothrombin conversion or direct inhibitors of thrombin function decrease PCF when blood samples are clotted by batroxobin and/or recalcification. The effects of heparin on PCF are pronounced at levels below those routinely used for anticoagulation (*see* Fig. 7A) *(44)*. At normal therapeutic heparin levels, 1 U/mL, the effects of heparin on PCF can be reversed with the same doses of protamine sulfate used to reverse anticoagulation *(39)*. However, at the very high doses of heparin used during CPB (4 U/mL), significant suppression of PCF may persist despite complete normalization of the activated partial thromboplastin time (aPTT) by protamine administration (*see* Fig. 7B). Heparin and dermatan sulfate also suppress PCF in a dose-dependent fashion *(47)*.

Low-Molecular-Weight Heparins

The effects of low-molecular-weight heparins (LMWH) parallel those seen with the unfractionated preparations. Increasing concentrations of these agents produce dose-dependent prolongation of the TGT and suppression of PCF. This occurs with all of the currently available preparations. The effects of Lovenox (Rhône-Poulenc Rorer Pharmaceuticals, Inc., Collegeville, PA are typical and are depicted in Fig. 7C. Although most of the effects of LMWHs on PCF are probably the result of decreased thrombin generation, the suppression of PCF correlates with increasing anti-Xa activity; LMWH may have additional effects. Work with several LMWH preparations with no antithrombin activity and minimal anti-Xa activity revealed that some preparations continued to have significant ability to suppress PCF *(46)*. The exact mechanisms for this activity have not been elucidated.

Direct Antithrombin Inhibitors

All direct antithrombin agents tested to date have demonstrated dose-dependent

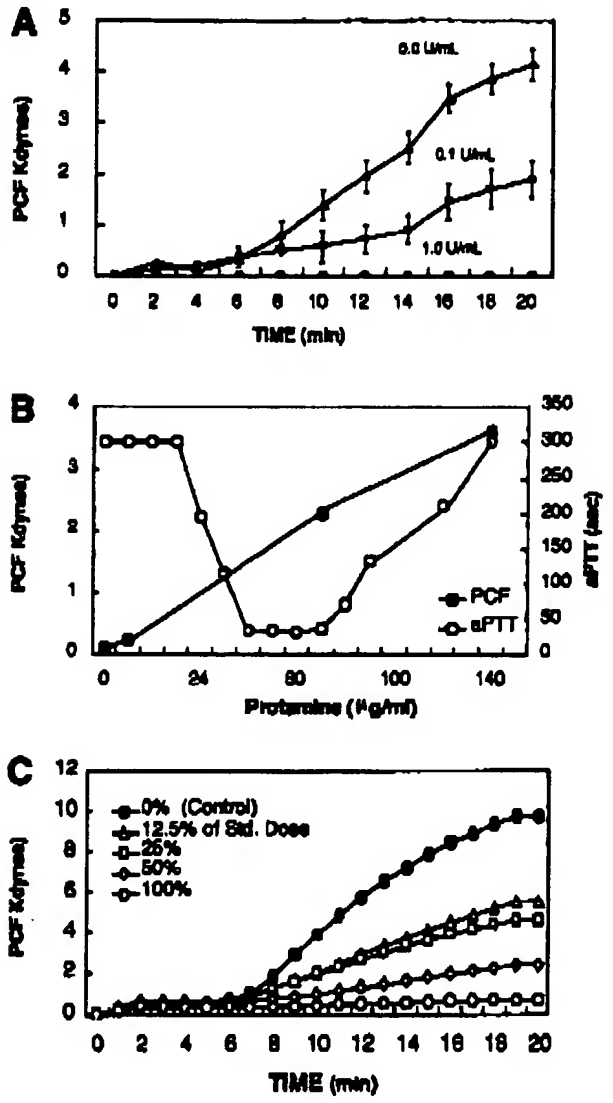

Fig. 7. (A) Effects of unfractionated heparin on PCF in whole blood. Clots were formed by the addition at time 0 of batroxobin and calcium to citrated whole blood. Final heparin concentrations are as indicated. (B) Effects of increasing protamine sulfate concentrations on the aPTT and PCF in blood containing high (4 U/mL) heparin concentrations. At high heparin concentrations, complete reversal of the anticoagulant effects of heparin may not ensure complete reversal of the antiplatelet effects. (C) Effects of increasing concentrations of the low-molecular-weight heparin Lovenox® on PCF in whole blood.

prolongation of TGT and suppression of PCF *(48)*. These agents include the physiologic inhibitors antithrombin III and thrombomodulin, the biologic agents hirudin and its derivatives (refludan), and synthetic antithrombins (PPACK, Thromstop, and Argatroban [Smith Kline Beecham Pharmaceuticals, Philadelphia, PA]).

Vitamin K Antagonists: Warfarin and Coumadin

These anticoagulants block the hepatic enzyme vitamin K epoxide reductase, reducing the liver's ability to recycle vitamin K. The subsequent relative deficiency of vitamin K results in the production of several clotting factors that lack important posttranscriptional modifications. Vitamin K serves as a cofactor in the addition of hydroxyl groups to specific glutamic acid residues. The addition of the hydroxyl groups allows these specific residues to bind calcium, which is critical to the formation of salt bridges. These bridges allow the formation of multimolecular complexes that allow the rapid activation of factor X and prothrombin. When warfarin is administered, clotting factors II, VII, IX, and X lack the modified glutamic acid residues and, as a consequence, thrombin generation is delayed and reduced. As expected, TGT is prolonged and PCF is reduced (*see* Fig. 5B). TGT and PCF can be normalized in such samples by the addition of recombinant activated factor VII (rVIIa).

EFFECTS OF MEDICATIONS ON PCF: ANTIPLATELET AGENTS

Aspirin

Aspirin is the most commonly used antiplatelet medication. At least part of its benefit is derived by its acetylation of cycloxygenase. Cycloxygenase is critical to the production of thromboxane A_2, which is a profound agonist of platelet function. Platelet secretion and aggregation are dramatically

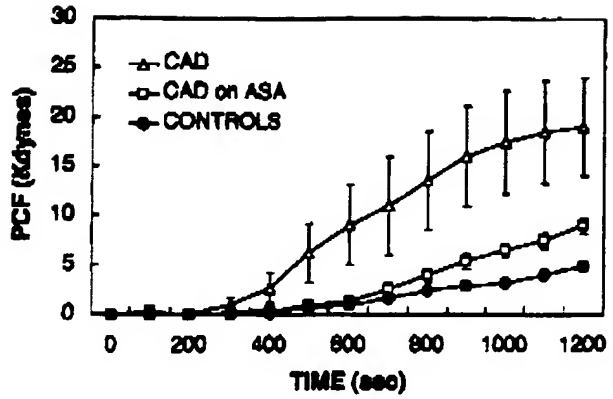

Fig. 8. PCF in normal controls (n = 10), in patients with CAD on aspirin (n = 8), and in patients with CAD who were not taking aspirin because of contraindications (n = 3). Clots were formed by the addition of thrombin and calcium to platelet-rich plasma at time 0.

altered by aspirin administration. When normal, asymptomatic volunteers are given one aspirin, less than a 5% change is noted in PCF *(51)*. However, in patients with coronary artery disease, the effects may be very different. When patients with coronary artery disease on or off aspirin are compared, the PCF values for those off aspirin were much higher. Although aspirin may be having a significant effect in these patients, it did not decrease the PCF into the normal range (*see* Fig. 8). This is consistent with results found in chest pain patients presenting to the emergency room. Although platelet aggregations were significantly depressed as a result of nonsteroidal anti-inflammatory agents, PCF remained significantly elevated *(52)*.

Intravenous GP IIb/IIIa Blockers

Abciximab

Abciximab (ReoPro®; Eli Lilly and Company, Indianapolis, IN) is the Fab fragment of the chimeric human–murine monoclonal antibody 7E3 against human glycoprotein IIb/IIIa *(63)*. It has great clinical utility in the treatment of acute coronary syndromes and is used routinely to prevent acute coronary artery stent thrombosis *(64,65)*. Because it binds to GP IIb/IIIa, abciximab totally ablates platelet aggregation. Because platelets use the same receptor to bind to fibrin during clot retraction and PCF development, abciximab produces a dose-dependent reduction in PCF *(66)*. If the clots are formed by the direct addition of thrombin, the amount of abciximab required to affect PCF is much higher than required to suppress aggregation. Indeed, aggregation is almost completely suppressed just as the PCF begins to decline. Although platelet aggregation is sensitive to low levels of Abciximab, PCF is sensitive to higher concentrations and declines across the therapeutic range (*see* Fig. 9A). This apparent phase shift in clinical response is the result of increased requirements for abciximab in thrombin-containing systems. In the presence of thrombin, platelets expose more GP IIb/IIIa in the appropriate format for binding of fibrin(ogen). Thus, more abciximab is required to ensure blockade. The PCF assay can be made sensitive to low-level ReoPro, by adding a known amount of GP IIb/IIIa-binding agent to the test sample or clotting reagent prior to the performance of the assay (*see* Fig. 9B). Monitoring of abciximab by PCF correlates with results seen in a modified thromboelastographic method *(67,68)*.

Tirofiban

Tirofiban (Aggrastat®; Merck & Co, Inc., West Point, PA) is a nonpeptide antagonist of GP IIb/IIIa that blocks platelet aggregation. It is indicated for the treatment of acute coronary syndrome, including patients who are to be managed medically and those undergoing PTCA or atherectomy *(69,70)*. As with abciximab, tirofiban produces a dose-dependent suppression of PCF. If the clots are formed by thrombin addition, more tirofiban is required to suppress PCF than to prevent platelet aggregation.

Eptifibatide

Eptifibatide (Integrilin®; COR Therapeutics, Inc., South San Francisco, CA) is a cyclic heptapeptide containing six amino acids and

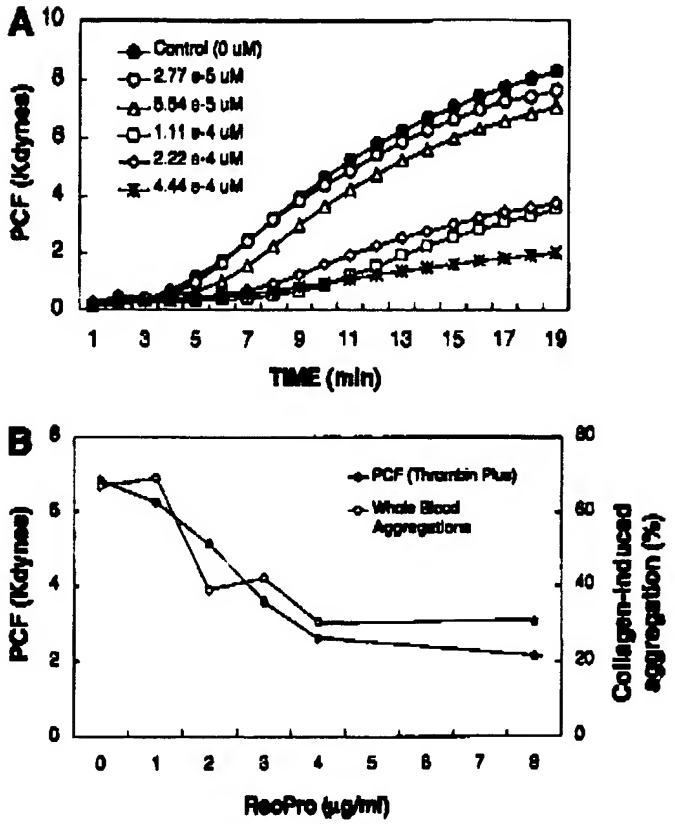

Fig. 9. **(A)** Effects of increasing concentrations of abciximab on the kinetics of PCF development in whole blood clotted by the addition of thrombin (1 NIH U/mL) and calcium (10 m*M*). **(B)** Altered PCF assay with enhanced sensitivity to low-level GP IIb/IIIa blockade. With the new reagent, the sensitivity of PCF parallels that of platelet aggregation.

one mercaptopropionyl residue. Eptifibatide reversibly binds to GP IIb/IIIa, inhibiting platelet aggregation in a dose-dependent manner. Eptifibatide is indicated for the treatment of acute coronary syndrome, including patients who are to be treated medically and those who are to undergo angioplasty (*71,72*). As with abciximab and tirofiban, eptifibatide produces a dose-dependent suppression of PCF. If the clots are formed by thrombin addition, more eptifibatide is required to suppress PCF than to prevent platelet aggregation. The relative ability of the three available intravenous GP IIb/IIIa blockers to suppress PCF is demonstrated in Fig. 10.

Oral GP IIb/IIIa Blockers (Ticlopidine and Clopidogrel)

Ticlopidine

Ticlopidine hydrochloride is a platelet aggregation inhibitor whose clinical indication is for reduction of stroke risk in patients who have suffered a stroke or who have symptoms consistent with transient ischemic attack. Ticlopidine produces time- and dose-dependent inhibition of platelet aggregation and secretion. Ticlopidine has virtually no effect on PCF in normal patients. Its effects on PCF in patients at risk for stroke have not been evaluated.

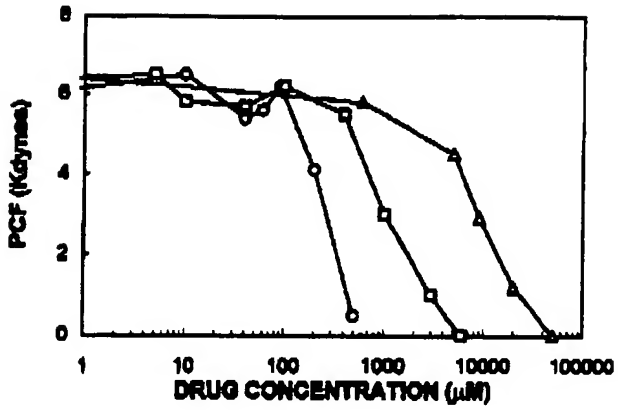

Fig. 10. Comparison of the potencies of abciximab (○), tirofiban (□), and eptifibatide (△) in the suppression of PCF.

Clopidogrel

Clopidogrel bisulfate (Plavix®; Bristol-Myers Squibb/Sanofi Pharmaceuticals, New York, NY) inhibits ADP-induced platelet aggregation by inhibiting ADP binding to its receptor. Inhibition of ADP binding inhibits ADP-mediated activation of GP IIb/IIIa. Clopidogrel is indicated for reduction of atherosclerotic events, including myocardial infarction, stroke, and peripheral artery occlusion. Clopidogrel has virtually no effect on PCF in normal blood. Its effects in patients with high PCF, such as patients with documented atherosclerosis, have not been evaluated.

EFFECTS OF MEDICATIONS ON PCF: MISCELLANEOUS AGENTS AND SUBSTANCES

Vitamin E

Vitamin E has mild antiplatelet properties. By itself, it has minimal effects on PCF. However, when used in combination with other antiplatelet medications, vitamin E can significantly augment PCF suppression. This has been demonstrated in a patient who had undergone angioplasty and stent placement for CAD. In the immediate postprocedure period, his PCF remained elevated despite the use of aspirin and clopidogrel. The addition of vitamin E caused additional lowering of the PCF into the normal range (*see* Fig. 11).

Aprotinin

Aprotinin is a broad-spectrum serine protease inhibitor with significant abilities to inhibit fibrinolysis. It has been used in coronary artery bypass grafting (CABG) surgery to reduce blood loss and transfusion requirements. In addition to its antifibrinolytic properties, aprotinin has two unique properties, as demonstrated by PCF analysis. First, low concentrations of aprotinin can increase PCF in normal platelets (*73*). Second, aprotinin can limit and/or reverse the inhibitor effects of heparin on PCF (*74,75*). These properties may help explain the postulated "platelet sparing" attributed to aprotinin. At high concentrations, aprotinin prolongs the TGT.

DDAVP

As noted in the case presented earlier, DDAVP increases PCF in acute renal failure. DDAVP also increases PCF and CEM, and it shortens the TGT in mild hemophiliacs who increase their factor VIII in response to DDAVP administration.

Intravenous Immunoglobulin

The administration of iv immunoglobulin can have effects similar to those seen in patients with elevated immunoglobulin secondary to multiple myeloma. Clot structures formed in the presence of high IgG levels are composed of thinner fibrin fibers and have increased CEM (*61*). In most cases, PCF is not altered.

Stem Cell Factor

Although the platelet has no nucleus, it has the highest concentration of stem cell factor receptors of any tissue. To this point, their function in platelets has not been defined. When stem cell factor is added to platelet-rich plasma, PCF is reduced by 80% (*76*). The

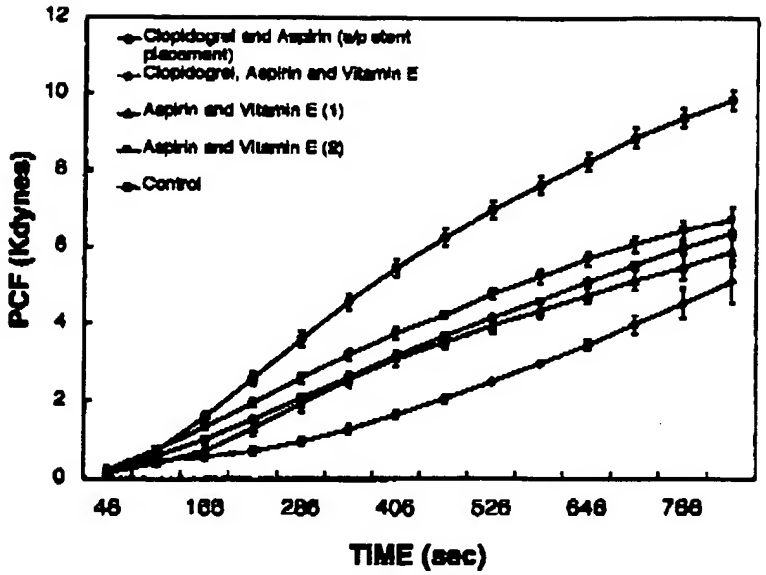

Fig. 11. Effect of vitamin E on PCF in a patient taking aspirin and clopidogrel status postangioplasty. Aspirin and Plavix curve was from blood 2 wk status postangioplasty. At that point, vitamin E was added and the other medications were continued. After an additional 2 wk, the aspirin–Plavix–vitamin E curve was obtained. After an additional month of therapy, Plavix was stopped. One month after stopping Plavix, the first aspirin–vitamin E curve was obtained. The second aspirin–vitamin E curve was obtained an additional 3 mo later.

mechanism by which this reduction occurs and its purpose are unknown.

EFFECTS OF ANTIFIBRINOLYTICS AS DEMONSTRATED BY THE TCLOT ASSAY

EACA

ε-Aminocaproic acid binds to the active site of plasmin and inhibits its proteolytic activity. It has been used in the treatment of mild bleeding disorders for a number of years. We have noted the ability of EACA to prolong the TCLOT in both normal blood and in patients with enhanced lytic rates.

Aprotinin

Aprotinin produces dose-dependent prolongation of the TCLOT. Its other effects were detailed earlier.

OTHER AVAILABLE MEASUREMENT TECHNIQUES

Several whole-blood platelet analysis modalities are currently available. Each differs significantly from the instrument detailed in this article. Although the differences have been detailed elsewhere (*77*), it is instructive to compare the PCF measurement to parameters measured by two of these technologies. The PFA-100® (Dade Behring, Deerfield, IL) measures a closure time for blood flowing through a capillary lined with a variety of agents. Closure occurs as a result of the adhesion of platelets to the capillary wall and subsequent aggregation of platelets onto the adherent platelets. The assay is sensitive to a variety of antiplatelet medications, to some forms of qualitative platelet dysfunction, and to von Willebrand disease. It is a nonclotting system and therefore does not assess platelet function in the presence of thrombin. Assay

time is less than 10 min and sample volume is 800 μL.

Thromboelastography is a whole-blood clotting assay that has significant utilization in cardiopulmonary bypass and other surgeries. Several instruments are currently available to perform this type of analysis (TEG®, Haemoscope Corp, Niles, IL; RoTEG®, Pentapharm GmbH, Munich, Germany). In these assays, clotting is detected and measured mechanically. Either the outer cup or an inner piston rotates during the measurement. As the clot forms between the surfaces of the piston and cup, coupling occurs and this alters the movement of the piston. A variety of parameters are measured that correlate with the time required for clotting to begin (*R*), the rate of clot development (α), and clot rigidity (MA). Although this assay is similar to the Hemodyne system in that it measures clot structure in whole blood, thromboelastography cannot measure PCF. Because elastic modulus is increased by PCF, MA will correlate to some degree with PCF (i.e., MA will increase with increasing PCF). However, MA is also sensitive to fibrinogen concentration (i.e., MA increases with increasing fibrinogen concentration). Thus, as a single parameter, it is difficult to know precisely what changes are causing changes in MA. Because PCF is relatively insensitive to fibrinogen concentration (27), the combination of PCF and CEM allows a degree of separation of clot structure from platelet effects. Assay times for the TEG and RoTEG are variable and can be extended to greater than an hour. The sample size is 340 μL for the TEG and 300 μL for the RoTEG.

LIMITATIONS OF THE CURRENT INSTRUMENTATION AND TECHNIQUE

A number of challenges and opportunities are inherent to the current measurement system. These include the expansion of the relatively limited sample throughput (three to four assays an hour), establishment of the most appropriate clotting agent, identification of the most appropriate sample anticoagulant, and establishment of the thrombotic and hemorrhagic risks associated with abnormal assay results. The problem of limited throughput can be most easily solved by development of a multichannel instrument. However, this may not be the most economical approach. Currently, a variety of clotting agents, including thrombin, batroxobin, calcium, tissue factor, tissue thromboplastin, kaolin, and so forth, are under investigation. Different clotting agents appear to bring out novel aspects of the measurement and may allow tailoring of the technique to a variety of clinical applications. For the short term, the primary assay will probably be initiated by simple recalcification or the addition of a combination of calcium and a snake venom enzyme. At present, the assay uses whole-blood samples anticoagulated with sodium citrate. Sodium citrate was used for simplicity, given the wide use of such samples for clinical coagulation testing. The most appropriate concentration of sodium citrate remains a question, and the potential for using other less platelet-altering anticoagulants such as corn trypsin inhibitor needs to be evaluated. Finally, the abnormalities of PCF, TGT, and CEM noted in patients with diseases known to have increased risk of hemorrhage (clotting factor deficiencies, cardiopulmonary bypass) and thrombosis (coronary artery disease, diabetes) are intriguing, but the direct establishment of the degree of risk associated with increasingly abnormal assay values needs to be established in clinical trials.

SUMMARY

The device and techniques outlined in this article offer promise in the detection and monitoring of conditions that place patients at risk for hemorrhage of thrombosis. The PCF assay offers a way to study platelets under conditions of maximal activation. The CEM parameter provides information about clot structural integrity. The TCLOT assay should facilitate the evaluation of potential abnormalities of

fibrinolysis by offering a simple way to screen for hyper fibrinolytic or hypofibrinolytic activity. The TGT is a simple way to screen for abnormal thrombin generation in the patient's whole blood. Although limited in nature, early studies appear to correlate with bleeding and thrombotic risk. Finally, each of these assays has the potential to monitor the effects of a wide variety of hemostatic and antithrombotic agents. It should be possible to screen for hemorrhagic risk by identifying patients with low PCF, low CEM, and prolonged TGT, and then monitor their response to hemostatic agents. It may also be able to screen for thrombotic risk by identifying patients with high PCF, high CEM, and short TGT, and then monitor their response to anticoagulant medications. Identification of enhanced fibrinolysis by a short TCLOT could be followed by monitoring normalization after the administration of antifibrinolytic agents such as EACA. Because the samples utilized in these assays are whole blood, it may be possible to define the contributions of monocytes and other white cells to normal and abnormal clot function. Further definition of the potential of these techniques awaits greater availability and utilization of the instrumentation.

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